

Objective Procedure for Fish Freshness Evaluation Based on Nucleotide Changes Using a HPLC System

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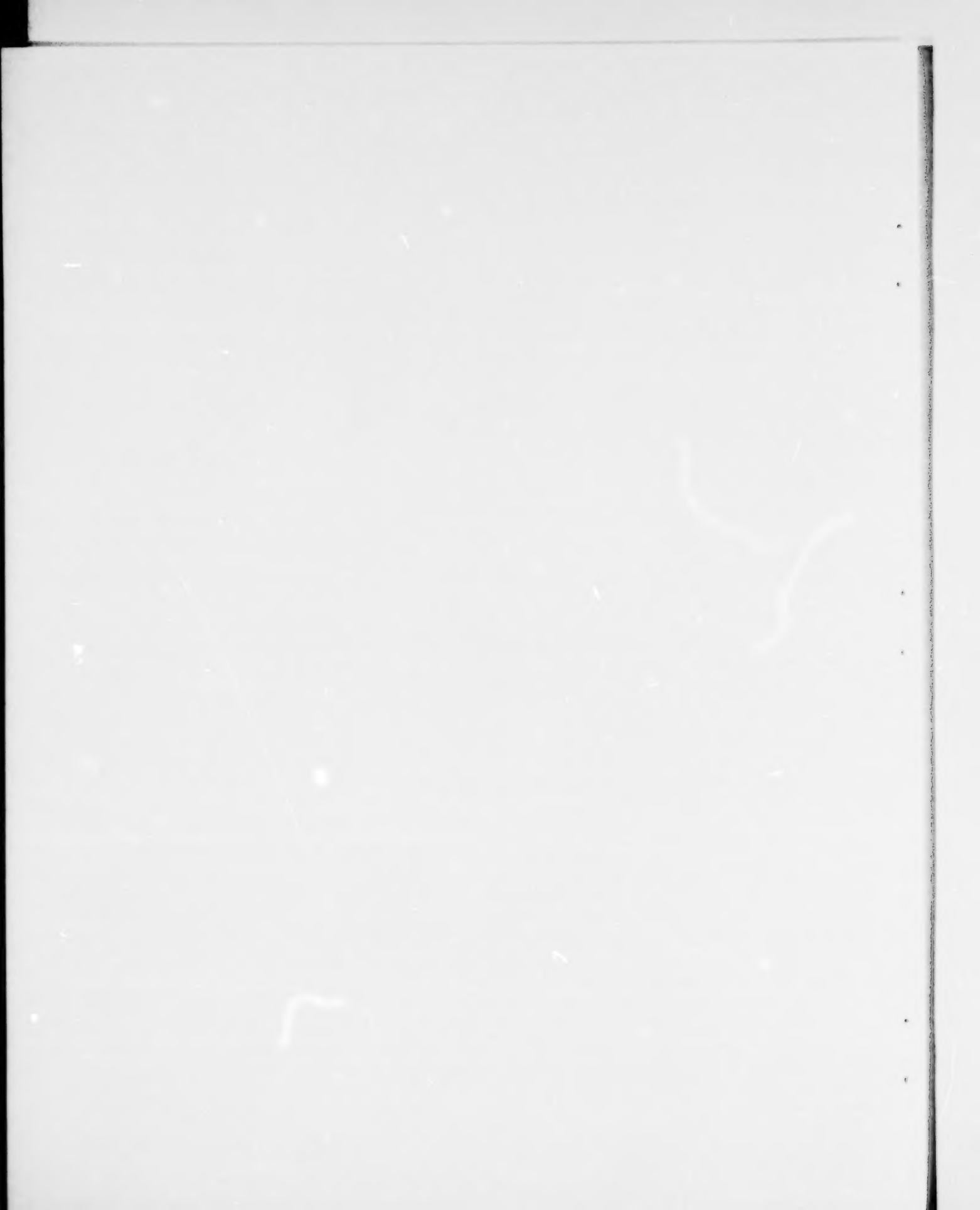
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OBJECTIVE PROCEDURE FOR FISH FRESHNESS EVALUATION
BASED ON NUCLEOTIDE CHANGES USING A HPLC SYSTEM

By

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ABSTRACT

B.G. Burns, P.J. Ke and B.B. Irvine, 1985. Objective Procedure for Fish Freshness Evaluation Based on Nucleotide Changes Using a HPLC System. Canadian Technical Report of Fisheries and Aquatic Sciences No. 1373, 39 Pages.

A high performance liquid chromatography (HPLC) method for determining ATP degradation products in biological samples has been developed. Nucleotides are extracted with 0.6 M perchloric acid, and determined by HPLC using a reversed phase microparticulate column with UV absorbance detection (254 nm). The mobile phase is a 0.01 M phosphate buffer (pH 4.5) at 0.5 ml/min.

By applying the described method, post-mortem nucleotide changes in various fish meats have been investigated. Two new indicators of fish freshness have been defined as $G = (Hx + INO) / (INO + IMP + AMP)$ and $P = (Hx + INO) / (INO + IMP + AMP + Hx)$ where IMP, AMP, Hx and INO are concentrations of inosine monophosphate, adenosine monophosphate, hypoxanthine and inosine respectively. Assays can be completed in less than 12 minutes and good comparative results were observed between G and P values and other more traditional quality indicators such as TVB, TBA, TMA and FFA. Biodegradation of post-mortem samples in terms of G and P values for a number of fish species have been discussed. Some tentative recommended guidelines of both P and G values have been proposed for further grading operations.

RÉSUMÉ

B.G. Burns, P.J. Ke et B.B. Irvine, 1985. Méthode objective d'évaluation de la fraîcheur du poisson basée sur la mesure de la variation de la teneur en nucléotides par CLHP. Canadian Technical Report of Fisheries and Aquatic Sciences. No. 1373, 39 Pages.

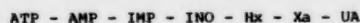
On a mis au point une méthode de chromatographie liquide à haute performance (CLHP) en vue de doser les produits de dégradation de l'ATP dans des échantillons biologiques. Les nucléotides sont extraits avec de l'acide perchlorique 0,6 M, puis dosés par CLHP sur une colonne de micoparticules à polarité de phase inversée, munie d'un détecteur à absorbance UV (254 nm). La phase mobile est constituée d'un tampon au phosphate 0,01 M (pH 4,5) s'écoulant à un débit de 0,5 mL/min.

Cette méthode a été appliquée pour mesurer, après la mort, la variation de la teneur en nucléotides de la chair de divers poissons. On a défini deux indices de fraîcheur, soit $G = (Hx + INO) / (INO + IMP + AMP)$ et $P = (Hx + INO) / (INO + IMP + AMP + Hx)$, dans lesquelles IMP, AMP, Hx et INO sont respectivement la concentration monophosphate d'inosine, de monophosphate d'adénosine, d'hypoxanthine et d'inosine. Les analyses nécessitent moins de 12 minutes. Elles permettent d'obtenir de bons résultats avec lesquels on peut comparer G, P et d'autres indices classiques de qualité, comme TVB, TBA, TMA et FFA. On discute de la biodégradation d'échantillons après la mort du poisson, en termes de G et de P pour un certain nombre d'espèces. On propose certaines directives provisoires relatives aux valeurs de P et de G au cours des opérations de classement.

INTRODUCTION

Uncontrolled enzyme protein degradation from both natural and bacterial sources occurs as a result of the failure of body regulators when fish die (Tanikawa *et al.*, 1970). Most past studies on fish freshness have been mainly based on the view that freshness is lowered by bacterial action. However, as fresh fish spoils, it passes through the following sequence of events: rigor mortis - dissolution of rigor mortis - autolysis. Therefore, freshness of fish must also be considered closely related to biochemical changes in fish before putrefaction, as fish will also spoil under aseptic conditions through natural enzyme degradation (Ehira, 1976). In particular autolytic and biochemical deteriorations in fresh fish become more important when proper chilling and handling are used.

Post-mortem nucleotide degradation in most fish muscle proceeds primarily via the following sequence of reactions:



where ATP = adenosine triphosphate, AMP = adenosine monophosphate, IMP = inosine monophosphate, INO = inosine, Hx = hypoxanthine, Xa = xanthine, and UA = uric acid. There are various stages in this degradative sequence which could be considered as indices of quality such as the dephosphorylation of IMP or the formation of Hx. The measurement of nucleotides and their breakdown products offer distinct advantages over the other objective chemical tests such as trimethylamine (TMA), dimethylamine (DMA), total volatile bases (TVB) and others which essentially measure bacterial spoilage (Martin *et al.*, 1978). For example, dephosphorylation of IMP is primarily autolytic (Jones 1965) and occurs during the period of early chilled storage. Hx accumulation in fish tissue reflects the initial phases of autolytic deterioration as well as later contributions through bacterial spoilage (Jones and Murray, 1962; Jones *et al.* 1964; Fraser *et al.*, 1968; Kassensarn *et al.*, 1963; Ehira, 1976). Hx concentrations have already been proven to correlate very well with eating quality in a number of fish species (Jones *et al.*, 1964; Fraser *et al.*, 1968; Spinelli, 1969; Beauchat, 1973; Hiltz and Dyer, 1970).

One of the most popular methods of analysis of nucleotides and their degradation products involves the use of selected enzymes. Hx has most often been analyzed by the enzymatic action of Xanthine oxidase which rapidly converts Hx to Xa and subsequently to UA (Figure 1) which is measured spectrophotometrically at 290 nm (Analytical Methods Committee, 1979). Modifications to the enzyme method have led to automation employing a redox indicator dye (Burt *et al.*, 1968), test paper strips (Jahns *et al.*, 1976), and colorimetric enzyme assay procedures (Beauchat, 1973). Simultaneous assay of nucleotides and their breakdown products has in the past centered mainly around ion exchange column chromatographic separation (Jones, 1960; Jones and Murray, 1964) followed by the spectrophotometric quantification of fractions or more recently measurements with enzyme sensors (Karube, *et al.*, 1984). Fraser *et al.*, 1967, also developed a rapid thin layer

chromatography (TLC) method, but the method was only semiquantitative. Most of these methods suffered either from non-specific measurements, laborious procedures, or poor reproducibility. With various modifications to reversed phase high performance liquid chromatography (HPLC) procedures (Anderson and Murphy, 1976; Wortheson *et al.*, 1980; Brown *et al.*, 1979) a rapid, simple and specific method for simultaneously determining the IMP, AMP, Hx and INO content in fish tissue samples has been developed (Burns and Ke, 1985).

The estimation of fish freshness is very important in the food industry both to the consumer and the processor. Proven parameters of fish quality such as FFA, TBA, TVB, TMA, DMA and pH values have already been used for various quality control operations but difficulties have been experienced from variations in reproducibility and correlations with eating quality for some species. We have applied the above HPLC method to the study of nucleotide changes in representative members of fresh groundfish, fatty fish and shellfish. Recommended G and P values have been applied to the quality grading of these species in an attempt to give more specific results in comparison with other quality parameters.

EXPERIMENTAL

PREPARATION OF REAGENTS

Hx, AMP, IMP, and INO were purchased in purified form (Sigma Chemical Company). Potassium phosphate (Baker), KOH, and perchloric acid (Fisher) were all ACS grade. Purified water (Omnisolv BDH) was used to prepare the HPLC mobile phase. Other chemicals used for various interference investigations were ACS grade.

- (1) **Extracting solvent:** 0.6 M perchloric acid. Add 32.3 ml of concentrated perchloric (60%) to a 500 ml volumetric and dilute to the mark with distilled water.
- (2) **Potassium hydroxide phosphate buffer pH 7.6:** Dissolve 8.16 g of KH_2PO_4 in approximately 60 ml of water and adjust the pH to 7.6 with 50% KOH. Dilute to 100 ml with distilled H_2O .
- (3) **50% KOH:** Dissolve 50 g of KOH in 50 ml of distilled water and cool to room temperature.
- (4) **HPLC Mobile Phase:** 0.01 M potassium phosphate buffer pH 4.5. Dissolve 1.36 g KH_2PO_4 in approximately 400 ml of purified water, adjust the pH to 4.5 with KOH or H_3PO_4 as necessary; dilute to 1L.
- (5) **Standards:**

Stock Solution: Individual standards are prepared by dissolving 0.010 g of Hx, INO, AMP, and IMP respectively in 40 ml of distilled water. Make up to the mark in 50 ml volumetrics. Keep individual standards frozen until required; thaw and dilute 1-10 before use.

Mixed Standards (Working Solutions): 2.5, 5.0, 7.5 and 10 µg/ml of IMP and Hx and 5.0, 10, 15 and 20 µg/ml respectively of AMP and INO. Pipette 0.125, 0.25, 0.375 and 0.50 ml of each stock solution of IMP and Hx into four separate 10 ml volumetric flasks. Add 0.25, 0.50, 0.75 and 1.0 ml respectively of each stock solution of AMP and INO to the above flasks. Make up to the mark with distilled water. The mixed standards solutions are stable for about 1 week when stored at 0-4°C.

APPARATUS

The apparatus used consisted of a Waters liquid chromatography system including two Model M 510 pumps, a Model U6K injector, a Model 660 solvent programmer, a Model 481 variable wavelength detector set at 254 nm, and a BBC Servogor 120 strip chart recorder. The chromatographic column was a RP-2 MPLC cartridge type (Brownlee labs) reversed phase analytical column (4.6 mm x 10 cm; 10 µm particle size) directly coupled to a 3 cm MPLC guard column packed with the same material. An RP-8 MPLC cartridge type column (4.6 mm x 10 cm; 10 µm particle size) was used for some of the work. The columns were held in a 13 cm cartridge system holder (Brownlee labs). A Virtis Model "23" blender and a Waters aqueous sample clarification kit were used for sample preparation.

RECOMMENDED PROCEDURE

(1) **Fish Sampling:** Atlantic cod (*Gadus morhua*) were obtained live from the Halifax Laboratory aquaria and were bled, gutted, and placed on ice immediately. Commercially caught cod and mackerel (*Scomber scombrus*) were obtained from local fresh fish retailers. The cod had been previously bled, gutted and held on ice. Mackerel obtained in the round state were gutted before being placed on ice. These fish were considered to be one day old upon arrival. Queen Crab (*Chionoecetes opilio*) were obtained live at dockside at Louisbourg, Nova Scotia and transported to the Halifax Laboratory by truck in an RSW system. Upon arrival the crab were bled and the crab sections placed on ice. Fish were sampled (at least two fish) at regular intervals throughout the holding experiments. Experiments ran up to 26 days. Samples were filleted or shucked and the meat samples homogenized in a food processor and stored at -40°C in 2 kg plastic bags until analysis.

(2) **Extraction and Cleanup:** Proceed as is outlined in Figure 2. Weigh 5 g of the frozen, finely chopped fish meat samples and place in the blender flask with 50 ml of 0.6 M perchloric acid. Blend for 2 minutes at maximum RPM. Suction filter flask contents through Whatman No. 1 filter paper using a small amount of distilled water to rinse the flask. Mix filtrate well and note volume carefully (V₁). Transfer 1.0 ml of the filtrate to a screw topped test tube

containing 1.0 ml of the potassium hydroxide phosphate buffer (pH 7.6). Mix the solution, cool to 0-4°C, then filter through a Waters aqueous clarification kit. Inject aliquots (V₂) of the neutralized filtrate directly into the HPLC for analysis. Dilute with distilled water if necessary (D).

(3) **HPLC Determination:** Set the flow rate at 0.5 ml/min (1.0 ml/min if using the RP-8 column) and let the column and detector equilibrate 20-25 minutes. Inject 10 µl aliquots of each mixed standard solution into the HPLC. Determine the absorbances of the various nucleotides from peak heights recorded at 254 nm. Plot peak height versus µg injected to provide a standard curve. Inject 10 µl aliquots of appropriately diluted sample extracts. Standard curves are prepared at least twice per day to assure accurate quantitation.

(4) **Calculations of G and P Values:** G and P values are based on the accumulation and/or degradation of Hx, IMP, AMP and INO. Hx content in fish tissue is calculated from the following equation:

$$\text{Hx content } (\mu \text{ moles/g}) = \text{KPKV}_1 \text{ D/HV}_2 \text{W} \quad (1)$$

where:

Pk = peak height (mm)

H = slope of standard curve (mm/µg)

V₁ = total volume of perchloric extract plus wash (ml)

D = Dilution factor of neutralized extract before HPLC

V₂ = injection volume on HPLC (µl)

W = weight of sample (g), and

K = 14.71 (µl) (µmole)/(ml) (µg) a constant which takes into account the 1:1 dilution during neutralization.

Based on our recommended procedures, the slope of the standard curve for Hx determination is 3130±130 (mm/µg), and the dilution factor (D), and injection volume (V₂) are 10 and 10 µl respectively. Thus, equation (1) can be simplified as equation (2):

$$\text{Hx content } (\mu \text{ moles/g}) = (6.97 \times 10^{-4})(\text{KPKV}_1/\text{W}) \quad (2)$$

IMP, AMP and INO content in fish tissues are calculated using the equation (1) but using the following K values: 5.75, 5.76 and 7.46 (µl)(µmoles)/(µg)(ml), respectively. Slopes must be recalculated if columns or experimental conditions are changes. G values are calculated from the following equation:

$$G = \frac{\text{Hx} + \text{INO}}{\text{INO} + \text{IMP} + \text{AMP}} \quad (3)$$

while P values may be calculated from:

$$P = \frac{\text{Hx} + \text{INO}}{\text{INO} + \text{IMP} + \text{Hx} + \text{AMP}} \quad (4)$$

Example: In a run the following data was obtained for a Day 1 aquarium cod sample containing Hx, IMP and INO.

Pk (mm)			H (mm/ μ g)			V ₁	V ₂	D	W
IMP	Hx	INO	IMP	Hx	INO	(ml)	(μ l)		(g)
104	23	29	1838	3125	1338	66	10	10	5.777

Calculations:

$$\text{Hx content } (\mu\text{moles/g}) = \frac{14.71 \times 23 \times 66 \times 10}{3125 \times 10 \times 5.777} = 1.24$$

$$\text{IMP content } (\mu\text{moles/g}) = \frac{5.75 \times 104 \times 66 \times 10}{1838 \times 10 \times 5.777} = 3.72$$

$$\text{INO content } (\mu\text{moles/g}) = \frac{7.46 \times 29 \times 66 \times 10}{1338 \times 10 \times 5.777} = 1.85$$

Therefore from equation (3) the G value is:

$$G = \frac{1.24 + 1.85}{1.85 + 3.72 + 0} = 0.55$$

and from equation (4) the P value is:

$$P = \frac{1.24 + 1.85}{1.85 + 3.72 + 1.24 + 0} = 0.45$$

OTHER TESTS

- (1) **pH:** pH was measured by direct insertion of a combined glass calomel electrode into the pooled fish homogenate in a manner similar to that described by Vyncke (1981).
- (2) **TVB:** TVB were estimated by the method of Woyewoda and Ke (1980) as modified from Cox and Pearson (1962) where the TVB were distilled into a 2% boric acid solution. The boric acid solution was then subsequently titrated back to its original pH.
- (3) **TMA:** TMA was estimated by the method of Dyer (1945) as modified by Tozawa (1971) where trimethylamine is colorimetrically determined at 410 nm as the picrate salt.
- (4) **TBA:** TBA values in lean samples were determined by a rapid direct spectrophotometric method using a monophasic reaction system as described by Ke and Woyewoda (1979). In the case of fatty samples, a direct distillation method was employed (Robles - Martinez *et al.*, 1982).
- (5) **FFA:** FFA were determined in tissues and lipids by a titrametric method using a ternary solvent system (CHCl_3 : MeOH: iPrOH) and with m Cresol purple as indicator (Ke and Woyewoda, 1978).
- (6) **Miscellaneous:** Moisture contents were determined on 5 g samples of homogenate placed in a vacuum drying oven at 60°C. Percent fat was determined as described by Ke and Woyewoda (1978).

RESULTS AND DISCUSSION

HPLC APPLICATION

The percent relative standard deviations (RDS %) of Hx, IMP, AMP and INO determined by the recommended HPLC method over a range of standards and fish sample concentrations is shown in Table 1. Replicate analysis of standard solutions indicates good reproducibility over the range of concentrations studied (Table 1) with variations of less than 7% in all cases.

Replicate HPLC analysis of homogeneous fish tissue samples from the initial sampling (day 0 or day 1) and from day 11 (cod and crab) and day 12 (mackerel) fish held on ice were also completed (Table 1). Average percentage relative standard deviations for Hx, IMP and INO contents were 6.17 $\pm$ 2.8 (range 3.3 in cod to 10 in crab); 4.95 $\pm$ 3.4 (range 1.5 in cod to 9.9 in cod) and 4.09 $\pm$ 3.0 (range 1.0 in cod to 8.7 in cod) respectively (Table 1). Unfortunately, AMP was not detected in any of the groups chosen indicating rapid conversion of ATP to IMP with no subsequent AMP build up.

Recoveries of nucleotides (0.5 - 12.5 μ moles/g) added to samples of cod, mackerel and crab were in the 90% range. This compares quite favorably with those reported by Worthesen *et al.* (1980) whose 0.5 μ mole Hx/g spike of whitefish muscle yielded recoveries of 92.5%.

IMP, Hx, AMP and INO are easily separated from one another and completely eluted from the 10 cm RP-2 reversed phase column using a 0.01 M potassium phosphate buffer (pH 4.5) at 0.5 ml/min within 12 minutes (Figure 3). If less than baseline separation is required, the analysis time can be shortened to approximately 5 minutes at 1.0 ml/min with adequate resolution. The RP-2 column is very stable with no loss of resolution or change in retention times experienced over a 2 month period of heavy usage. Highly reproducible linear standard curves were obtained (Figure 4) over the range of concentrations used. ATP and ADP were not well separated from each other or the solvent front and were therefore not included in the mixed standard. Fortunately ATP is rapidly degraded to IMP during or shortly after the death struggle by the partial dephosphorylation and deamination of ATP (Jones and Murray 1964; Martin *et al.* 1978). The measurement of peak heights for quantitation were quite reproducible with 6-9 replicate injections for each level of 25, 50, 75 and 100 ng/ injection showing maximum variations of 6.40, 1.37, 5.47 and 4.45% for Hx, INO, IMP and AMP respectively. Retention times were very stable showing variations of less than 4%. Detection limits for Hx and IMP were 5 ng and for AMP and INO, 10 ng.

DEVELOPMENT OF G AND P VALUES AS QUALITY INDICES

While useful indices of freshness could be based on individual nucleotide or breakdown products accumulation or disappearance, an indicator that would incorporate the measurement of several of these nucleotides would be advantageous. Multiple measurements remove some sample to sample and species to species variations

with only a slight loss in sensitivity. Ehira (1976) has described a K value based on the concentrations of various nucleotides and their breakdown products estimated from the following formula:

$$K = \frac{INO + Hx}{ATP + ADP + AMP + IMP + INO + Hx} \times 100 \quad (5)$$

while Karube *et al* 1985 described a K_1 value as:

$$K_1 = \frac{INO + Hx}{IMP + INO + Hx} \times 100 \quad (6)$$

Both of these values are based mainly on the appearance and disappearance of IMP and describe a period of early chill storage not measured by such objective chemical tests as the TMA test. In fact, by the time substantial amounts of TMA accumulate, fish are in incipient stages of spoilage (Spinelli *et al* 1964; Jones 1965). The most serious limitation of such an indicator is that the reaction is completed well within the edible storage life of a number of fish species (Kassensarn *et al* 1973; Jones *et al* 1964).

Now a freshness estimation index G is proposed which is calculated from equation 3. The G value is based on the accumulation of Hx but also reflects the disappearance of IMP, AMP and INO. This index is useful over the entire iced shelf-life of the fish studied.

A second quality indicator P calculated from equation (4) serves as an indicator of spoilage during the early stages of chill storage.

The RSD% of P and G values determined for cod, mackerel and crab samples are shown in Table 2. Variations were less than 3% for P values while variations of less than 2.9% were recorded for G values (Table 2). G and P values would appear to be effective in reducing some of the sample to sample variation noted above.

Tests for interferences from various bio-substances possibly present in fish tissue were carried out by adding up to 50 mg of each compound to a 5 g fish sample. All of the compounds listed in Table 3 did interfere slightly with the described method giving relative deviations of about 5% or less for P values and 10% or less for G values.

LABORATORY TESTS FOR QUALITY ASSESSMENT

Tables 4-7 contain summaries of the results of all objective quality indicators determined for aquarium held cod, commercial cod, mackerel and queen crab respectively. Figures 5-8 illustrate the degradation patterns of the chosen nucleotides, TMA, TVB and TBA changes in aquarium held cod (Group A), commercially caught cod, mackerel and queen crab respectively. The changes of pH and FFA during the icing shelf-life studies of the various fish species tested are shown in Figures 9 through 12 while G and P values are shown in Figures 13-16 and 17-20 respectively.

Post-mortem Nucleotide Degradations in Fish

Aquarium held cod day 0 samples were very fresh with samples being taken less than one hour

after death. This is reflected in the nucleotide degradation pattern shown in Figure 5. Initial levels of IMP are quite low at 0.451 μ moles/g rising to 5.06 μ moles/g on day 2 (Table 4). This is dissimilar from levels reported for trawl caught cod which usually show high initial levels of IMP as a result of ATP degradation during the death struggle (Jones and Murray 1962, 1974; Martin *et al* 1978). Inosine levels are also quite high initially at 4.70 μ moles/g which is the highest level reached. INO levels then fall rapidly to 2.08 μ moles/g on day 2 (Table 4) then rise to almost initial levels then gradually tapering off to 0.132 μ moles/g on day 22. Hx levels are initially low but rise rather steadily throughout the course of the experiment up to day 22 (Table 4). After day 22 Hx levels begin to tail off. These patterns (Figure 5) with the expectation of the initially high INO levels are very similar to those described for relaxed cod by Fraser *et al*, 1967 and Jones and Murray, 1961.

Group B cod (Table 4) were essentially a repeat of the early stages of relaxed cod nucleotide degradation with daily samplings up to day 8. An almost identical pattern emerged with IMP peaking at 24 hours (Table 4) then gradually falling off to 0.394 μ moles/g (Table 4) indicating that perhaps cod naturally contain fairly high levels of INO. AMP was not detected in either of the two groups of relaxed cod indicating rapid conversion of ATP to IMP with no subsequent AMP buildup.

Commercially caught cod showed a very similar degradation pattern (Figure 6) to that described for aquarium held cod, especially after 3-4 days holding period on ice (Figure 5, 6). Commercially caught cod were considered to be one day old at the time of the first sampling. Therefore, the initial values would be expected to vary slightly from those of the aquarium held cod. INO and IMP for the most part were at their highest on day 1, IMP did show a further increase on day 3 (Table 5) and gradually tapered off while Hx values increased steadily over the sampling period (Figure 6, Table 5). The most significant difference between the aquarium held and commercially caught cod was that the degradations and/or accumulations occurred more quickly (Figure 6, Table 5) indicating a shorter shelf-life for the commercially caught cod. This no doubt reflects a less stringent handling, washing and icing regime for a commercially caught fish versus laboratory handled fish.

Nucleotide degradation patterns for mackerel were distinctly different from those of the cod sampled. INO levels were initially 4.18 μ moles/g (Table 6) and remained fairly consistent up to day 19 on ice (Figure 7) where they fell to 1.09 μ moles/g. IMP levels began at 2.74 μ moles/g on day 1, increased to 3.13 μ moles/g on day 3 then tapered off to 0.896 μ moles/g by day 10 (Table 6) of the holding period. Hx, in general, steadily increased throughout (Figure 7, Table 6). Values obtained were quite similar to those previously reported for mackerel iced immediately after catching (Fraser *et al* 1968).

Queen crab were held for only eleven days on ice because severe blackening of the sections would by this time render the product unsaleable in any case. Very foul odors were also in evidence by this time. IMP and INO values were at

their maximum on day one on ice (Figure 8, Table 7) and gradually tapered off to 0.018 and 0.529 μ moles/g respectively by day 9 (Table 7). Hx initial levels of 0.289 μ moles/g (Table 7) increased generally throughout the course of the experiment (Figure 8). Maximum values of IMP, Hx and INO obtained for Queen crab throughout the holding period were less than one half those experienced by either mackerel or cod.

Comparison of G and P Values with Other Quality Indicators

- (1) Cod: Fillets from aquarium held cod used in the experiment contained $0.471 \pm 0.022\%$ (range 0.454 - 0.518%) fat and $81.5 \pm 1.4\%$ (range 79.5 - 84.2%) moisture (Table 4). Commercial cod fish fillets contained $0.481 \pm 0.014\%$ (range 0.448 - 0.500%) and $81.1 \pm 0.65\%$ (range 80.0 - 82%) moisture (Table 5).

Initial TVB values for aquarium held cod were relatively steady in the 22 mg-N/100g range up to day 14 where values tend to dramatically increase up to 75.4 mg-N/100 g on day 26, the final sampling day (Table 4). This rise closely parallels the rise in G value over the same period (Figure 5, 13) and marks a progressed state of fish spoilage. Values of over 30 mg-N/100g are indicative of spoiling fish while values approaching 50 mg-N/100 g indicate spoiled fish (Pearson 1973; Woyewoda and Ke 1980). That is in a 3 grade system such as proposed for squid (Ke et al 1979) the aquarium held fish would be considered Grade F (unacceptable) around day 17 by physical grading and TVB data (Figure 5). A similar pattern of results were observed for commercially caught cod (Figures 6, 14, Table 5) but the fish were judged unacceptable by physical and TVB data at day 10.

The onset of bacterial spoilage is noted in the aquarium held cod by a rise in the TMA values starting around day 8 (Figure 5, Table 4). This initial rise corresponds to a leveling off of P values (Figure 17) which occurs at approximately day 8. There is also a rapid increase in TMA values up to 38.6 mg TMA-N/100g beginning around day 15 (Figure 5, Table 4) corresponding to the previously mentioned rise in G values. It has been proposed that the maximum allowable TMA values be set between 5-10 mg/100g of tissue for international trade (Martin et al 1978). Dyer and Mounsey (1945) judged cod and haddock unacceptable when concentrations of 10-20 mg/100g tissue had been reached. Woyewoda and Ke (1980) set levels of less than 3, 3-10 and greater than 10 for grades A, B and F grade squid respectively.

Similar patterns are in evidence for the commercially caught cod (Figures 6, 14, 18, Table 5) with the onset of bacterial spoilage in evidence starting around day 4 with a corresponding rise in P values. A more dramatic increase starts around day 6 corresponding to a rise in G values.

Although both TBA values (Figures 5, 6) and FFA (Figures 9, 10) generally increase over the course of the icing experiment, values associated with spoilage as a result of

oxidative rancidity were never reached (Robles-Martinez et al 1982). pH (Figures 9, 10) also remained relatively constant over the course of the experiment.

- (2) Mackerel: Mackerel fillets used in the experiment contained $4.01 \pm 1.5\%$ (range 0.656 - 5.73%) fat and $72.1 \pm 2.3\%$ (range 67.8 - 75.5%) moisture (Table 6).

TVB values remain almost constant up to day 14 at around 30 mg-N/100g then begin a fairly steady increase (Figure 7). TMA values begin a similar increase at about the same time (Figure 7). This is about the spot where the P value begins to plateau (Figure 19). This plateau tends to reflect the disappearance of IMP which according to Fraser et al (1968) is at or near the unacceptability level for mackerel. In any event sharp increases in either TMA or TVB values indicates a progressed state of protein degradation in the species under study. The pattern for the G values closes resembles that of either the TVB or TMA values in that the initial values remain relatively constant up to days 12-14 where a steady increase begins (Figure 15, Table 6).

TBARS values have been correlated with taste panel data and the recommended guidelines for assessing rancidity development in mackerel and other fatty fish have been established (Robles-Martinez 1982). Quality grades have been established as 0-8, 9-20 and over 21 μ moles/kg fish for excellent, good and unacceptable grades. Mackerel in this study would be judged unacceptable around day 5 as a result of TBA values (Table 6, Figure 7).

FFA increased throughout the course of the experiment. FFA accumulate as a result of enzymatic fat hydrolysis. The increase in FFA in mackerel tissue as a function of holding time is depicted in Figure 11. Although cut off limits have not been assigned for various quality grades, FFA would appear to be a useful quality test for mackerel. pH as previously remains relatively constant (Figure 11) over the course of the experiment.

- (3) Queen Crab: Queen crab used in this experiment contained $1.27 \pm 0.11\%$ (range 1.13-1.43%) fat and $86.6 \pm 1.1\%$ (range 85.3-87.6%) moisture (Table 7).

TVB, TMA and TBA values all show a fairly sharp increase beginning around day 4 (Figure 8). Limits of acceptability for TBA and TVB are reached around day 5-6 (Table 7, Figure 8). These rises closely parallel a rise in the G values (Figure 16, Table 7) and a plateauing of the P values (Figure 20, Table 7). Blackening of the exposed meat in the crab sections and objectionable odors have also reached an unacceptable level by this time.

FFA also rise steadily thorough the course of the experiment and may also be useful as a quality indicator (Figure 12). pH values remain relatively constant over the course of the experiment (Figure 12).

RECOMMENDED OBJECTIVE PROCEDURE FOR FISH FRESHNESS EVALUATION

The recommended G and P values for the quality assessment of fresh fish derived from comparisons with such chemical tests as TVB, TMA, TBA and FFA as well as physical evaluation are shown in Table 8. These values serve only as a guide and may be adjusted to meet needs or requirements if a higher quality product is desired. G and P values should not be used as the only parameters to evaluate fish quality. Additional indicators such as TBA, TVB, etc., should be used in order to have reliable quality assessments.

- (1) Cod: the G value is most useful as an indicator of spoiled fish, i.e. establishing the line between TB and TF grade fish. If suggested values of 10 mg TMA-N/100g and 45 mg-N/100g for TMA and TVB respectively are accepted as the cut off point for acceptable fish, then a G value of less than 5 would give a similar indication (Figures 5, 13).

It is much more difficult to establish a value for TA-TB grade fish. The disappearance of IMP has been correlated with a loss of fresh fish flavour in some species (Fraser *et al* 1968; Spinelli *et al* 1969; Jones and Murray, 1961) and may be a good TA/TB grade indicator for these species. For example, IMP dephosphorylation occurs between 8-12 days in chill stored cod (Jones 1963). For this reason the P value may be a good indicator of early loss of fish quality (TA/TB). If TMA levels of less than 3 and TVB values of less than 30 (Woyewoda and Ke, 1980) are accepted as being close to the TA/TB dividing line, aquarium held cod would go from TA/TB grade between day 10-14. If a P value of greater than 0.85 is accepted as the cut off point for TA/TB grade fish, then the fish would pass from TA-TB grade at approximately day 8. If a G value of greater than 2 is taken as the cut off point, fish would pass from A-B grade between days 10-14.

- (2) Mackerel: The most useful indicators of mackerel eating quality are those tests which measure the oxidative rancidity or enzymatic hydrolysis of fats contained in the mackerel flesh such as the TBA or FFA tests. G and P values might prove useful in the assessment of mackerel quality however, but only as indicators of protein degradation rather than actual eating quality. G values of greater than 1.5 and/or P values of greater than 0.95 would indicate an advanced state of protein degradation and would provide information similar to that provided by the TVB and TMA test.
- (3) Queen Crab: G and P values can prove useful in establishing the line between TB and TF grade crab. If a P value of greater than 0.9 and G value of greater than 1.5 are used as cut off points for acceptable crab then a holding time of approximately 6 days on ice is indicated for this experiment (Figures 16, 20). This is in close agreement with TBA, TVB, TMA and physical data (Figures 8, 12). There appears as yet to be no acceptable point for establishment of a G or P value which would indicate the TA/TB grade transition for queen crab.

CONCLUSIONS

Overall either the G value alone or a combination of G and P values should provide an excellent starting point for the establishment of a grading system. G and P values can be chosen for a 3 grade TA (excellent), TB (good) and TF (reject) system for cod and to establish the TB/TF transition point for queen crab. G and P values can establish the degree of protein degradation in mackerel but are not useful indicators of eating quality. Tests that measure the degree of oxidative rancidity (TBA) an enzymatic hydrolysis (FFA) are more useful indicators of eating quality for mackerel. Both G and P values are easily and quickly calculated from available data and could be easily programmed into a small microcomputer. The method also provides a valuable alternative test in the monitoring of fish quality.

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Table 1 Hx, IMP, AMP and INO content in various standards and fish samples as determined by the recommended HPLC method.

Sample	Hx Content ($\mu\text{Moles/g} \pm \text{RSD}\%$)	IMP Content ($\mu\text{Moles/g} \pm \text{RSD}\%$)	AMP Content ($\mu\text{Moles/g} \pm \text{RSD}\%$)	INO Content ($\mu\text{Moles/g} \pm \text{RSD}\%$)
Standard ^a	0.020 $\pm$ (6.5) 1.50 $\pm$ (2.3)	0.021 $\pm$ (5.5) 1.58 $\pm$ (3.3)	0.36 $\pm$ (5.0) 3.00 $\pm$ (4.5)	0.044 $\pm$ (1.6) 3.31 $\pm$ (1.4)
Crab ^b	0.289 $\pm$ (10) 1.07 $\pm$ (4.3)	0.870 $\pm$ (3.3) 0.0141 $\pm$ (2.5)	--- ---	1.60 $\pm$ (4.8) 0.781 $\pm$ (1.4)
Mackerel ^b	0.914 $\pm$ (6.5) 1.27 $\pm$ (8.9)	2.74 $\pm$ (4.1) 0.787 $\pm$ (8.4)	--- ---	4.18 $\pm$ (2.8) 4.89 $\pm$ (5.9)
Cod ^b	1.31 $\pm$ (3.3) 2.66 $\pm$ (4.0)	5.07 $\pm$ (1.5) 0.124 $\pm$ (9.9)	--- ---	2.06 $\pm$ (1.0) 1.87 $\pm$ (8.7)

a. Standard concentrations are μ moles/ml with 6 determinations done per standard
b. 3 determinations

Table 2. RSD% of P and G values calculated for various fish samples as determined by the recommended HPLC method.

Sample		P Value $\pm$ RSD%	G Value $\pm$ RSD%
Crab	1	0.680 $\pm$ (3.0)	0.759 $\pm$ (1.6)
	2	0.993 $\pm$ (0.036)	2.28 $\pm$ (1.1)
Mackerel	1	0.651 $\pm$ (0.11)	0.736 $\pm$ (0.95)
	2	0.887 $\pm$ (0.32)	1.09 $\pm$ (0.65)
Cod	1	0.923 $\pm$ (0.17)	1.06 $\pm$ (0.55)
	2	0.973 $\pm$ (0.10)	2.27 $\pm$ (2.9)

Table 3. Various interferences from some biocompounds in fish tissue on the recommended HPLC determination.

Compound	Amount added (mg)	G Value	% of variation	P Value	% of variation
Control*	---	2.25	---	0.804	---
DMA	5	2.35	+ 4.4	0.762	- 5.2
CuSO ₄	50	2.01	- 11.0	0.790	- 1.7
NaCl	50	2.12	- 5.8	0.800	- 0.5
Stearic Acid	5	2.20	- 2.2	0.816	+ 1.5
Cysteine	5	2.28	+ 1.3	0.807	0.37
TBHA	5	2.45	+ 8.9	0.837	+ 4.1
TBHQ	5	2.38	+ 6.2	0.832	+ 3.5
TMAO	50	2.09	- 7.1	0.789	- 2.0
Hemoglobin	5	2.09	- 7.1	0.802	- 0.25
Cystine	50	2.06	- 8.4	0.784	- 2.5
FeSO ₄	1	2.46	+ 9.3	0.829	+ 2.5
Sucrose	50	2.49	+ 11.0	0.834	+ 3.8
Palmitic Acid	5	2.39	+ 6.3	0.828	+ 3.1
Lactic Acid	50	2.28	+ 1.4	0.818	+ 1.9
MnSO ₄	5	2.38	+ 5.8	0.833	+ 2.9
Sorbitol	5	2.13	- 5.3	0.799	- 0.62
TMA	5	2.25	0.0	0.819	+ 1.9
Lecithin	50	2.45	+ 8.9	0.835	+ 4.0
Thiourea	5	2.29	+ 1.7	0.819	+ 1.8

*Samples of 5 g of cod tissue were used as the control and in all interference investigations.

Table 4. Objective quality evaluation of aquarium held cod.

	HELD IN ICE (DAYS) ^a												
Objective Quality Indicator	0	1	2	3	4	6	8	11	14	18	22	26	
TVB (mg-N/100g)	21.6	-	21.4	-	24.8	-	22.3	25.2	29.2	47.8	72.1	75.4	
TMA (mgTMA-N/100g)	0	-	0	-	0.391	-	0.492	3.76	4.03	14.0	38.6	26.6	
FFA(μ Mole/g tissue)	4.16	-	4.21	-	4.23	-	4.38	4.55	4.81	5.31	9.48	7.67	
FFA (μ Mole/g oil)	920	-	919	-	928	-	960	1002	1027	1101	1934	1478	
TBA (μ Mole/g oil)	0.704	-	0.640	-	0.711	-	0.747	0.830	0.905	1.30	2.82	0.962	
TBA(μ Mole/kg fish)	3.09	-	2.93	-	3.43	-	3.85	4.56	4.52	6.42	14.2	5.03	
pH	7.1	-	7.1	-	7.2	-	6.9	6.9	7.0	7.0	7.2	7.1	
Moisture content%	81.7	-	79.5	-	82.0	-	79.9	80.6	82.0	81.6	82.3	84.2	
Percent Fat	0.456	-	0.456	-	0.456	-	0.456	0.454	0.469	0.482	0.49	0.518	
IMP (μ Mole/g) ^b A _C B	0.451	-	5.06	-	3.83	-	0.174	0.124	0.091	0.133	0.157	0.145	
	0.197	4.07	3.54	2.41	0.394	0.165	0.074	-	-	-	-	-	
Hx (μ Mole/g) A	0.731	-	1.31	-	1.29	-	1.63	2.66	2.14	4.44	5.43	4.51	
B	1.28	1.20	0.984	1.16	2.61	1.74	2.73	-	-	-	-	-	
AMP (μ Mole/g) A	N/D ^d	-	N/D	-	N/D	-	N/D	N/D	N/D	N/D	N/D	N/D	
B	N/D	N/D	N/D	N/D	N/D	N/D	N/D	-	-	-	-	-	
INO (μ Mole/g) A	4.70	-	2.08	3.36	4.56	-	2.85	1.87	2.01	1.02	0.137	0.054	
B	4.62	1.64	2.43	-	4.03	3.87	1.89	-	-	-	-	-	
G value A	1.05	-	0.474	-	0.611	-	1.48	2.28	1.98	4.74	18.9	23.5	
B	1.22	0.500	0.571	0.783	1.50	1.39	2.44	-	-	-	-	-	
P value A	0.923	-	0.401	-	0.604	-	0.963	0.974	0.979	0.977	0.973	0.968	
B	0.968	0.411	0.491	0.652	0.945	0.971	0.985	-	-	-	-	-	

a. At least two cod fillets were pooled for each sampling day.

b. Group A cod were held 26 days and sampled on days indicated.

c. Group B cod were held 8 days and sampled on days indicated.

d. Not detected.

Table 5. Objective quality evaluation of commercially caught cod

Objective Quality Indicator	HELD IN ICE (DAYS) ^a										
	1	3	5	7	9	13	15	17	19	21	
TVB(mg-N/100g)	29.3	24.5	30.3	37.6	43.9	51.4	74.2	81.2	78.2	77.3	
TMA(mg TMA-N/100g)	0.502	0.848	7.77	14.3	16.6	32.9	43.7	36.0	40.0	37.3	
FFA(μ Mole/g tissue)	5.10	5.91	4.58	6.76	7.81	7.56	8.59	8.54	8.65	7.54	
FFA(μ Mole/g oil)	1040	1184	1018	1442	1673	1572	1806	1743	1788	1554	
TBA(μ Mole/g oil)	0.522	0.805	0.847	1.52	2.23	2.69	3.94	3.33	3.55	2.72	
TBA(μ Mole/g fish)	2.76	3.98	3.82	7.43	10.5	13.1	19.0	16.4	17.3	13.3	
pH	7.2	7.2	7.1	7.0	7.2	6.9	7.0	7.1	7.2	7.0	
Moisture Content%	80.0	80.4	81.0	81.3	82.1	81.9	81.6	80.7	81.1	80.9	
Percent Fat	0.490	0.500	0.448	0.483	0.467	0.481	0.476	0.491	0.484	0.485	
IMP(μ Mole/g)	1.88	3.33	0.499	0.157	0.133	0.135	0.189	0.176	0.238	0.102	
Hx(μ Mole/g)	1.11	0.946	2.70	3.49	3.26	4.63	4.53	3.76	4.48	4.51	
AMP(μ Mole/g)	N/D ^b	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	
INO(μ Mole/g)	3.99	3.12	2.85	2.07	1.61	0.819	0.222	0.134	0.115	0.193	
G value	0.869	0.631	1.66	2.50	2.80	5.71	11.6	12.6	13.0	16.0	
P value	0.731	0.550	0.918	0.973	0.974	0.976	0.962	0.957	0.951	0.979	

a. At least two cod fillets were pooled for each sampling day.

b. Not detected.

Table 6. Objective quality evaluation of commercially caught mackerel

Objective Quality Indicator	HELD IN ICE (DAYS) ^a										
	1	3	6	8	10	12	14	16	19	22	
TVB(mg-N/100g)	30.0	30.9	33.8	33.1	30.9	29.7	36.6	56.8	41.2	52.1	
TMA(mg TMA-N/100g)	0.545	0.767	1.79	2.38	4.20	3.65	17.9	41.9	35.2	38.2	
FFA(μ Mole/g tissue)	9.67	10.2	14.2	10.2	23.3	20.9	23.5	26.9	27.1	32.5	
FFA(μ Mole/g oil)	1475	389	288	178	552	498	595	478	604	977	
TBA(μ Mole/g oil)	0.963	0.158	1.15	0.785	1.23	3.16	1.50	1.69	1.07	1.27	
TBA(μ Mole/g fish)	6.32	4.17	60.5	45.0	51.8	133	59.4	66.6	48.1	42.3	
pH	6.9	7.1	6.8	7.0	7.1	7.2	7.4	6.7	6.8	6.8	
Moisture Content%	75.5	73.4	73.7	67.8	73.6	70.2	70.4	71.3	73.5	71.8	
Percent Fat	0.656	2.64	5.25	5.73	4.23	4.20	3.95	5.62	4.49	3.33	
IMP(μ Mole/g)	2.74	3.13	1.54	1.37	0.896	0.787	0.342	0.384	0.250	0.221	
Hx(μ Mole/g)	0.914	0.703	1.19	1.02	1.73	1.27	2.00	2.53	2.59	2.75	
AMP(μ Mole/g)	N/D ^b	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	
INO(μ Mole/g)	4.18	3.85	4.75	4.57	4.48	4.89	3.89	2.83	1.09	0.834	
G value	0.742	0.652	0.944	0.941	1.16	1.08	1.39	1.66	2.74	3.43	
P value	0.650	0.592	0.794	0.803	0.874	0.887	0.945	0.933	0.936	0.942	

a. At least two mackerel fillets were pooled for each sampling day.

b. Not detected.

Table 7. Objective quality evaluation of commercially caught Queen Crab.

Objective Quality Indicator	HELD IN ICE (DAYS)				
	1	4	7	9	11
TVB(mg-N/100g)	30.0	34.4	73.0	52.6	73.8
TMA(mgTMA-N/100g)	0.832	1.00	15.7	10.2	11.3
FFA(μ Mole/g Tissue)	4.14	3.78	6.17	6.58	7.85
FFA(μ Mole/g Oil)	292	303	506	522	696
TBA(μ Mole/g Oil)	0.153	0.210	8.62	8.08	8.60
TBA(μ Mole/g Fish)	2.19	2.86	105	101	97.1
pH	7.1	6.9	6.8	7.0	7.0
Moisture Content%	85.4	85.3	87.2	87.6	87.4
Percent Fat	1.43	1.30	1.22	1.25	1.13
IMP(μ Mole/g)	0.870	0.615	0.038	0.018	0.014
Hx(μ Mole/g)	0.289	0.348	0.901	1.63	1.07
AMP(μ Mole/g)	N/D ^a	N/D	N/D	N/D	N/D
INO(μ Mole/g)	1.60	1.44	0.728	0.529	0.781
G Value	0.764	0.869	2.13	3.94	2.33
P Value	0.684	0.743	0.977	0.991	0.992

a. Not detected.

Table 8. Tentatively recommended guidelines for quality assessment of fresh fish using G and P valves.
A = excellent, B = good, F = reject.

Fish	Grade	G Value	P Value
Cod	TA	< 2	< 0.85
	TB	2 - 5	N.A.*
	TF	> 5	N.A.
Mackerel ^a	TA or TB	N.A.	N.A.
	TF	> 1.5	> 0.95
Queen Crab ^b	TA or TB	< 1.5	< 0.9
	TF	> 1.5	> 0.9

*N.A. = not applicable

- a. G and P valves for mackerel are indicators of the state of protein degradation but do not necessarily reflect the eating quality. A fish with a G valve of <1.5 may still be rejected on the basis of rancidity.
- b. It is not possible to distinguish between A and B grade crab with G and P valves.

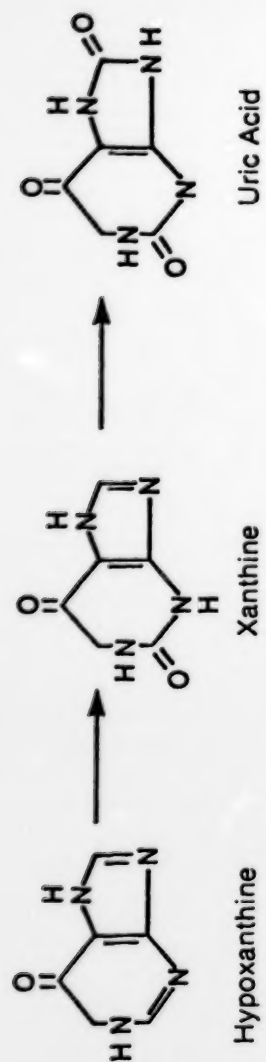


FIGURE 1: REACTION PATHWAY FOR THE XO CONVERSION OF Hx TO UA

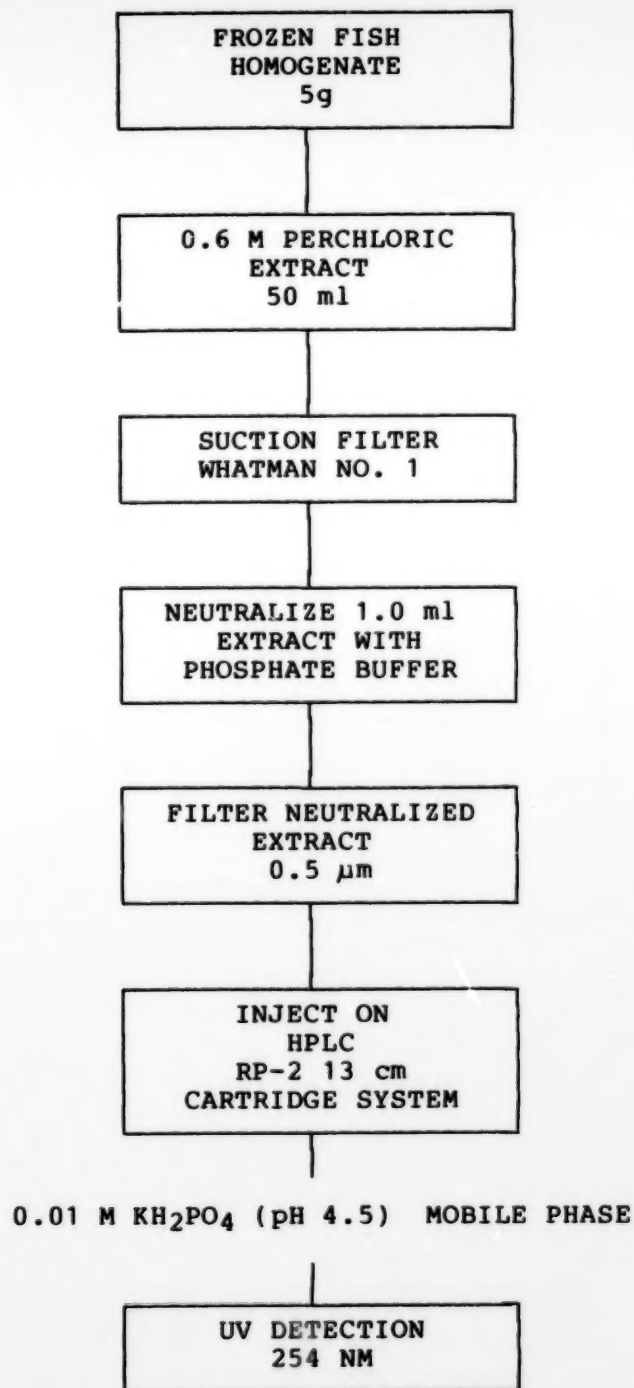


Figure 2.

Flow sheet of the extraction and cleanup of fish samples for HPLC analysis of nucleotides and their degradation products.

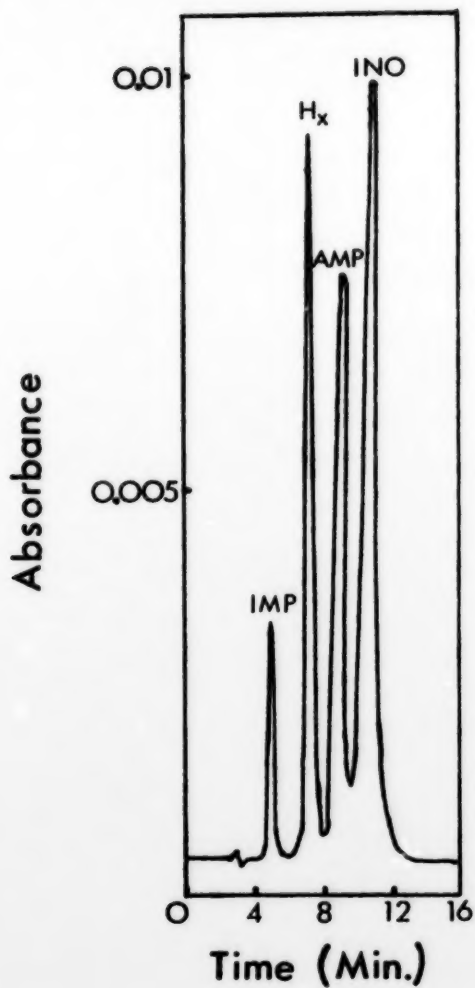


FIGURE 3: HPLC UV (254nm) TRACE OF THE SEPARATION OF STANDARD IMP, Hx (50NG EACH), AMP AND INO (100NG EACH) RUN ON A BROWNLEE MPLC RP-2 (10UM, 4.6MMID X 10CM, DEVELOPED WITH 0.01 M KH_2PO_4 BUFFER, PH 4.5 AT 0.5ML/MIN) REVERSED PHASE ANALYTICAL COLUMN.

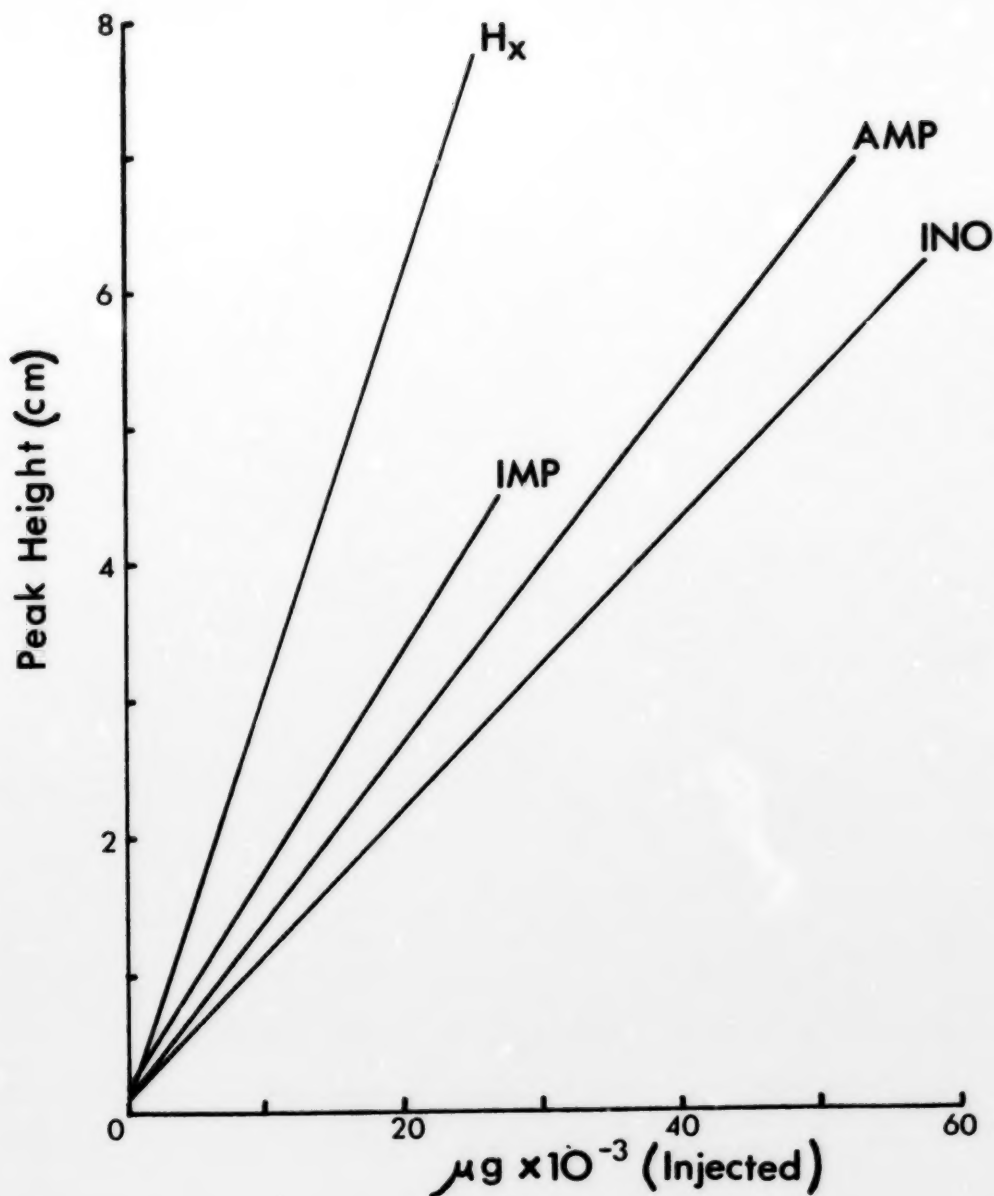


FIGURE 4: STANDARD CURVES FOR NUCLEOTIDE DETERMINATIONS AT 254 NM USING Hx, IMP, AMP AND INO AS STANDARDS.

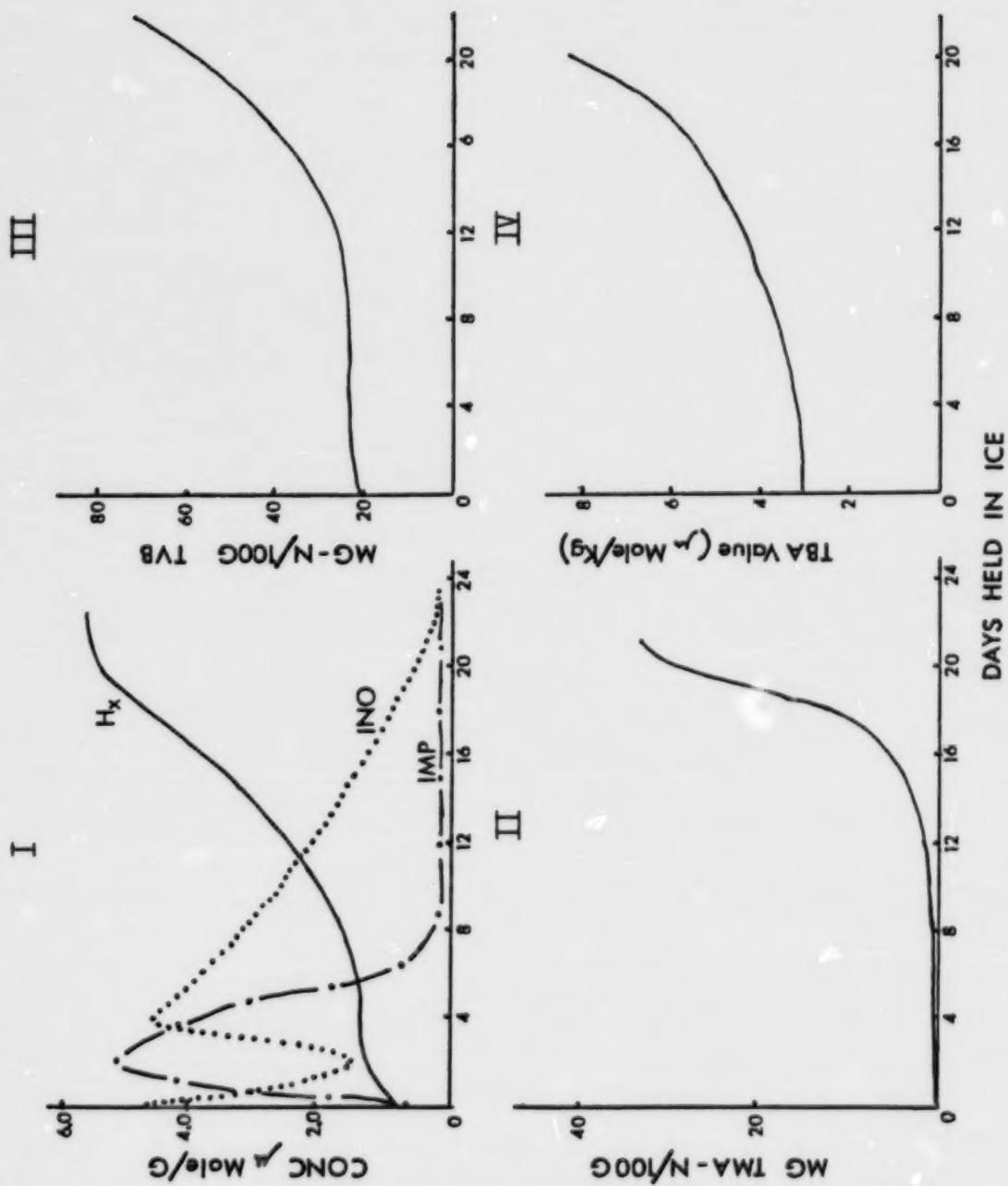


FIGURE 5: I. NUCLEOTIDE; II. TMA, III. TVE AND IV. TBA CHANGES FOR AQUARIUM COD (GROUP A) BLED, GUTTED AND HELD ON ICE.

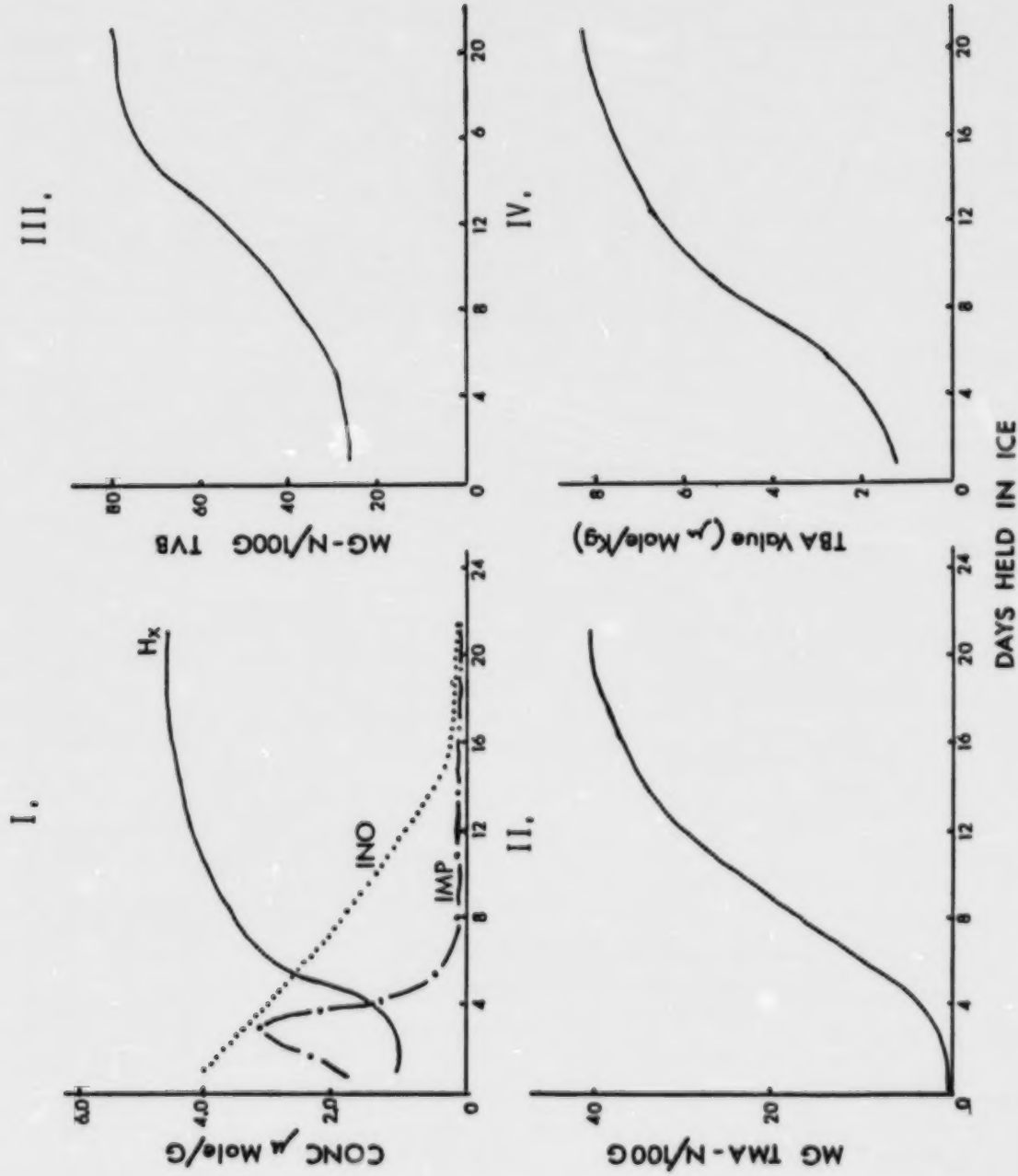


FIGURE 6: I NUCLEOTIDE; II TMA, II TVB AND IV TBA CHANGES FOR COMMERCIALLY CAUGHT COD BLED AND GUTTED AT SEA AND SUBSEQUENTLY HELD ON ICE.

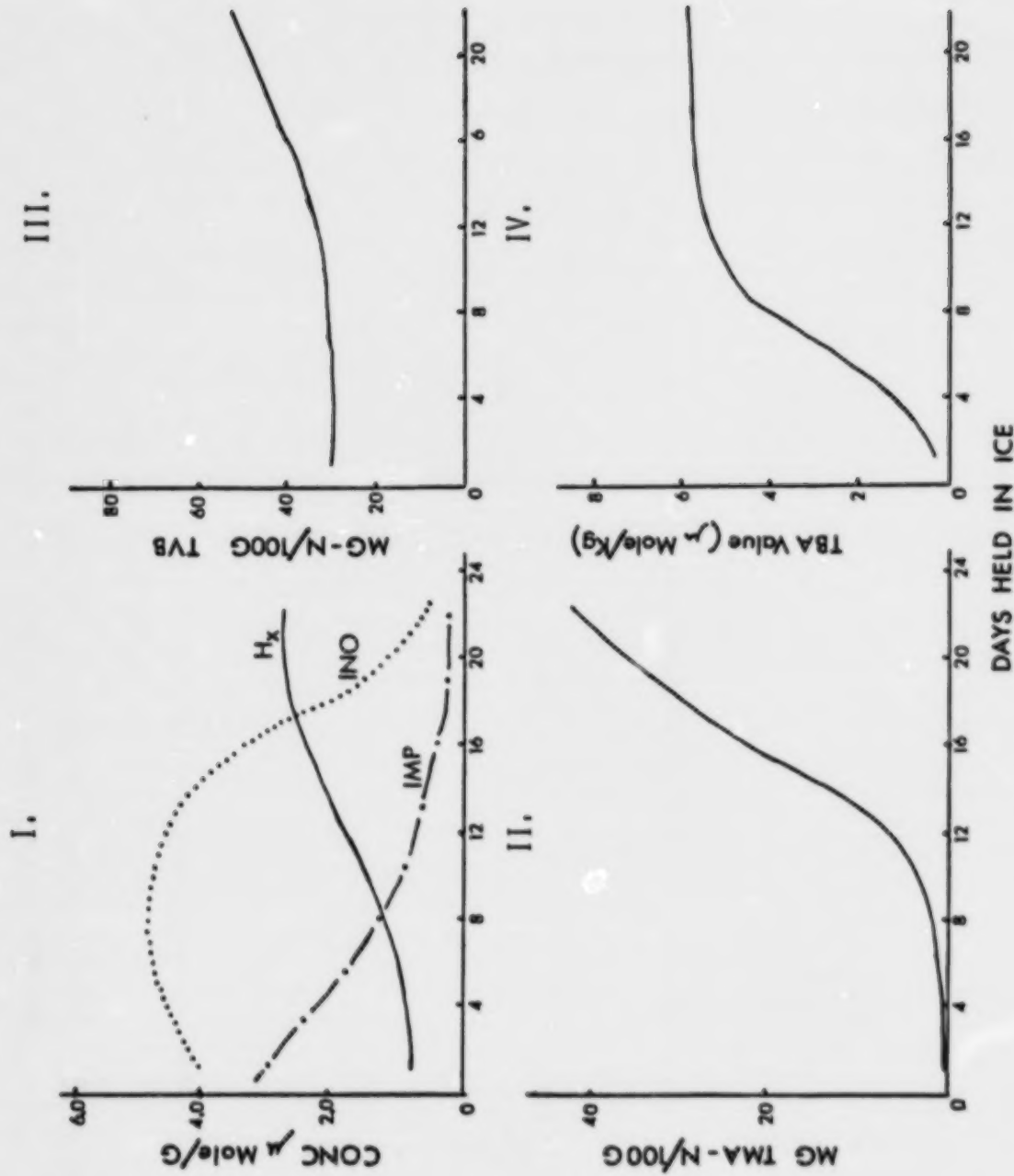


FIGURE 7: I NUCLEOTIDE; II TMA, III TVB AND IV TBA CHANGES FOR MACKERAL LANDED ROUND THEN GUTTED AND HELD ON ICE.

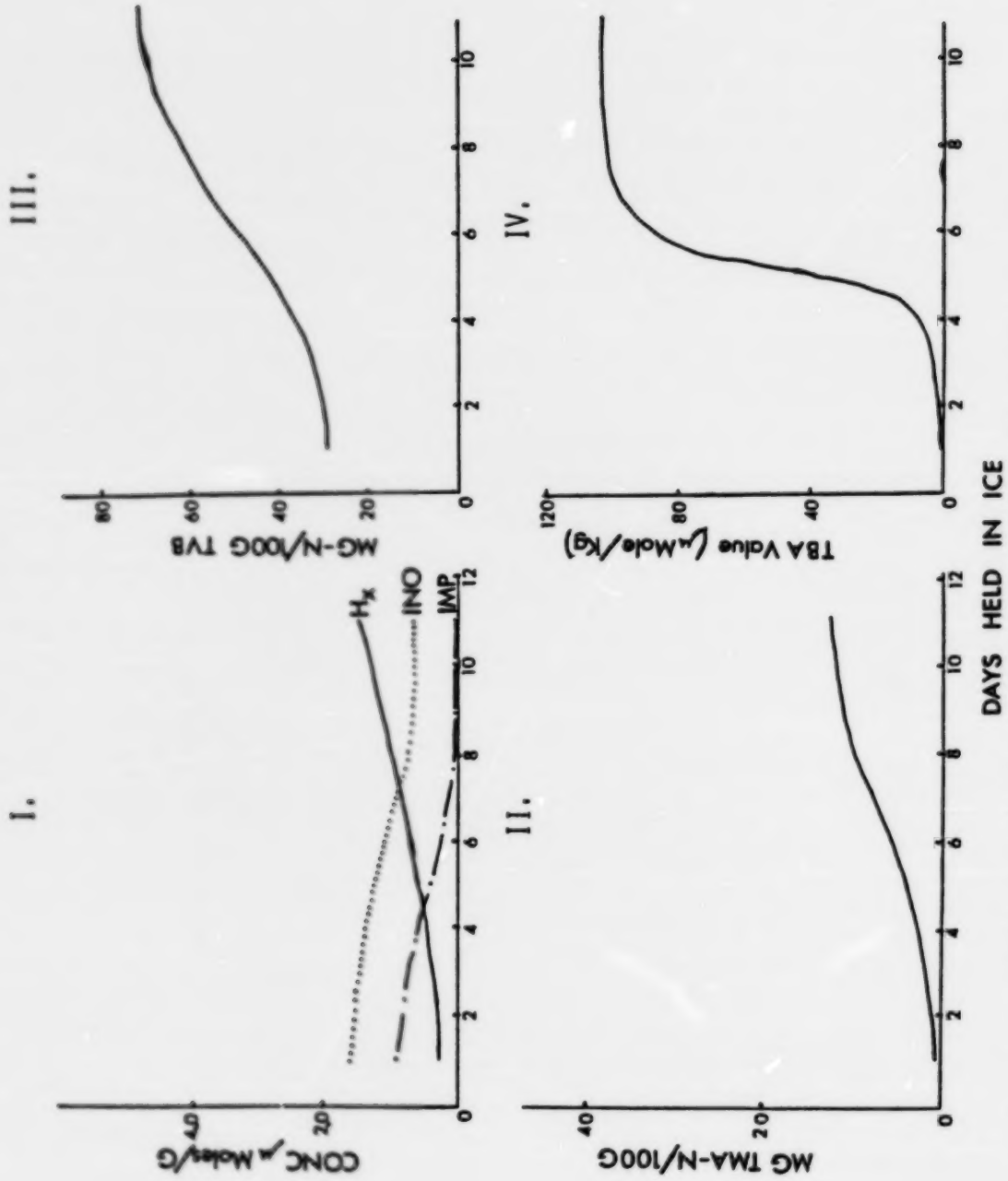


FIGURE 3: I NUCLEOTIDE; II TMA, III TVB AND IV TBA CHANGES IN SECTIONS FROM LIVE BATCHED QUEEN CRAB HELD ON ICE.

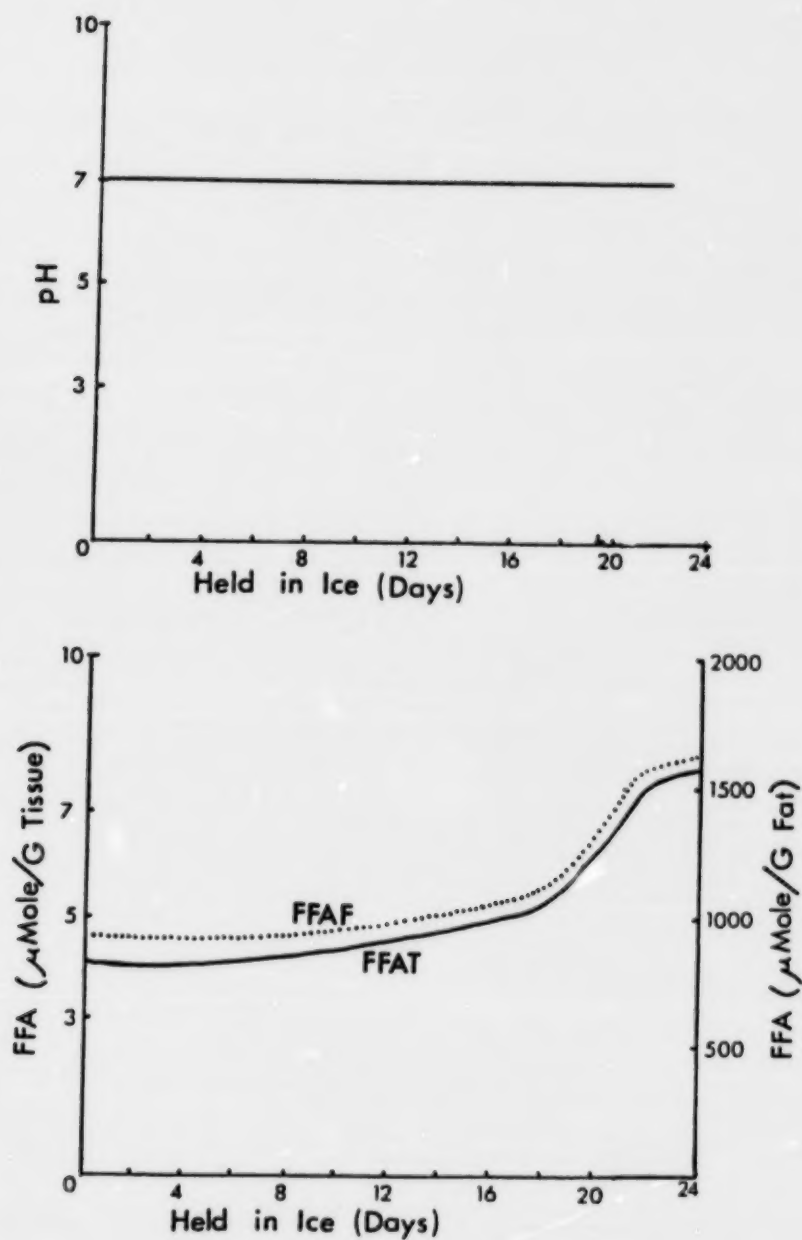


FIGURE 9: pH AND FFA (TISSUE AND FAT) CHANGES FOR AQUARIUM COD, (GROUP A) BLED, GUTTED AND HELD ON ICE.

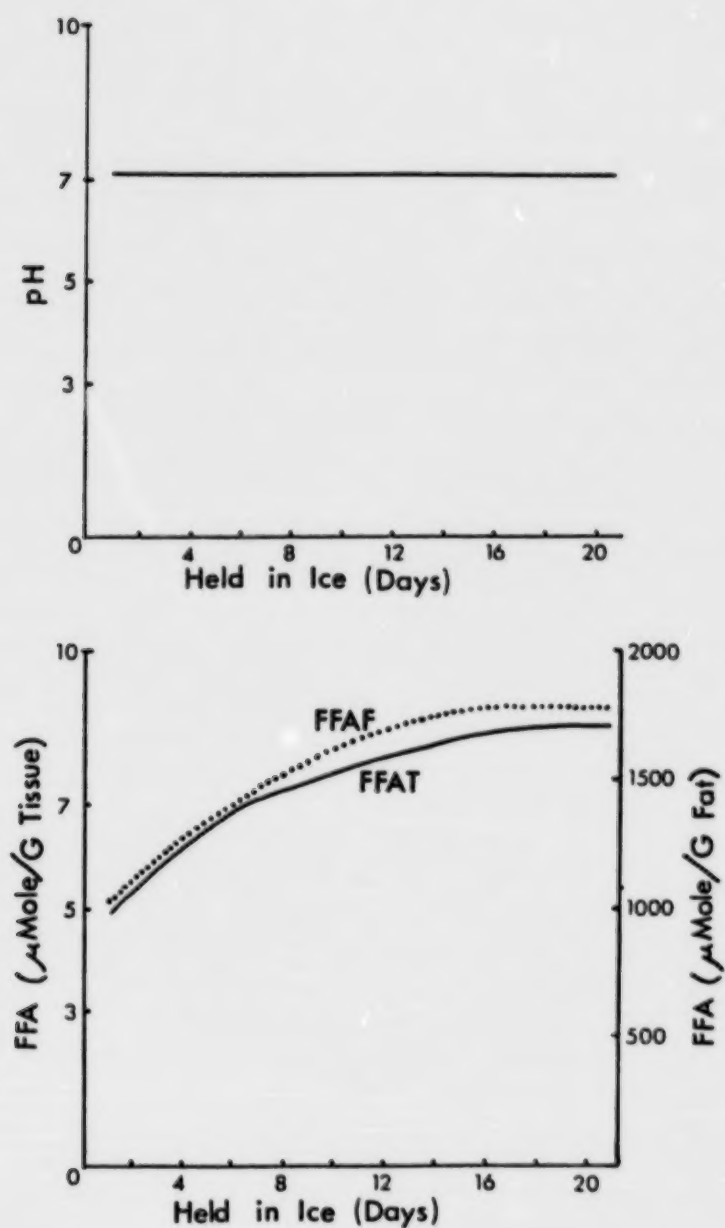


FIGURE 10: pH AND FFA (TISSUE AND FAT) CHANGES FOR COMMERCIALY CAUGHT COD, BLED AND GUTTED AT SEA AND SUBSEQUENTLY HELD ON ICE.

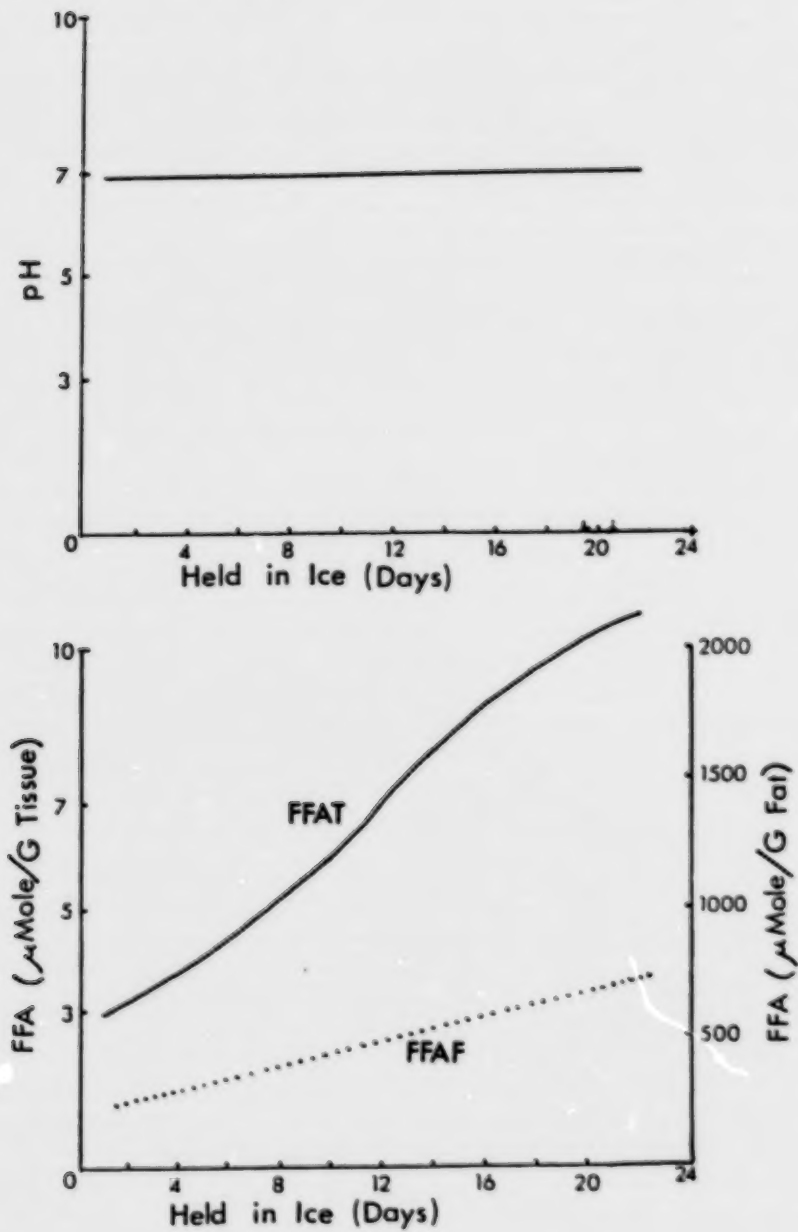


FIGURE 11: pH AND FFA (TISSUE AND FAT) CHANGES FOR MACKEREL LANDED ROUND THEN GUTTED AND HELD ON ICE.

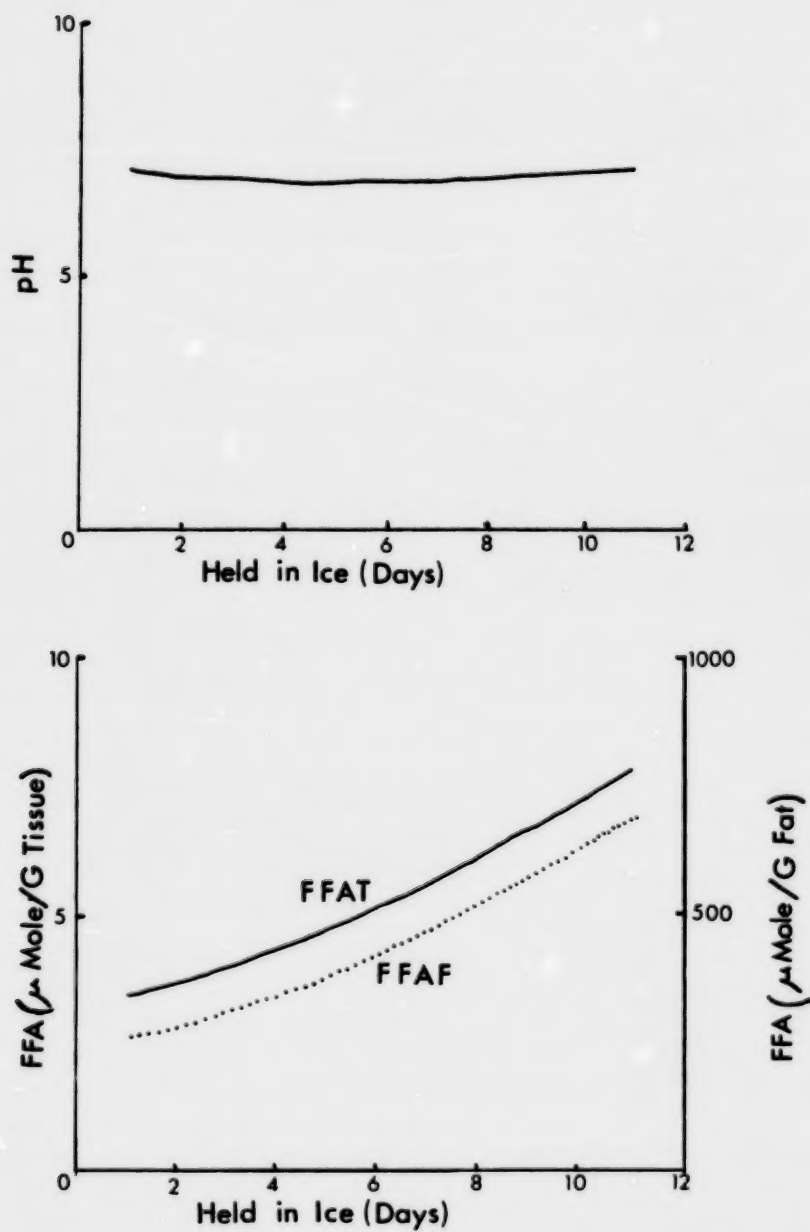


FIGURE 12: pH AND FFA (TISSUE AND FAT) CHANGES IN SECTIONS FROM LIVE BOTCHED QUEEN CRAB HELD ON ICE.

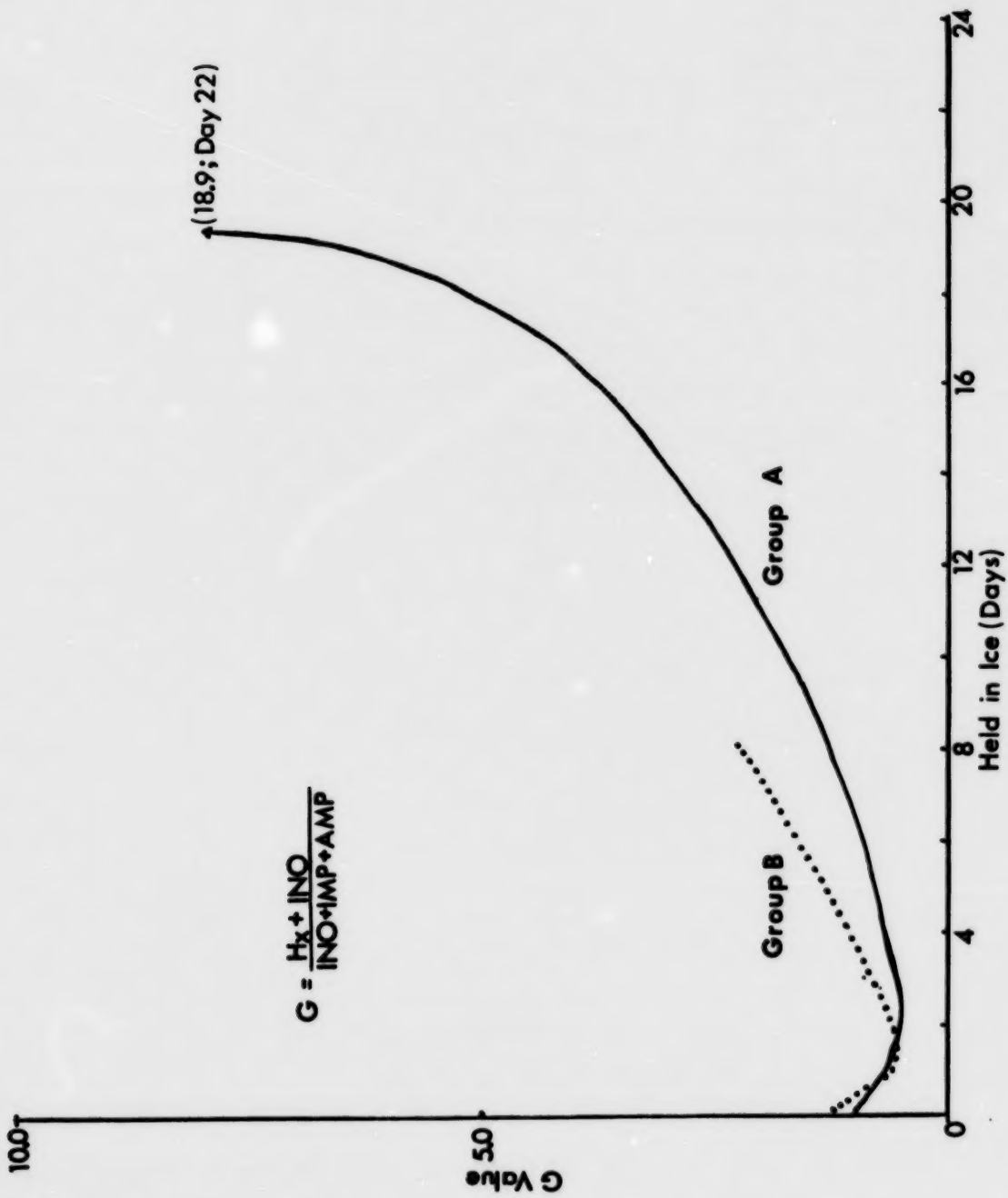


FIGURE 13: G VALUE CHANGES FOR AQUARIUM COD GROUP A, AND GROUP B, BLED, GUTTED AND HELD ON ICE.

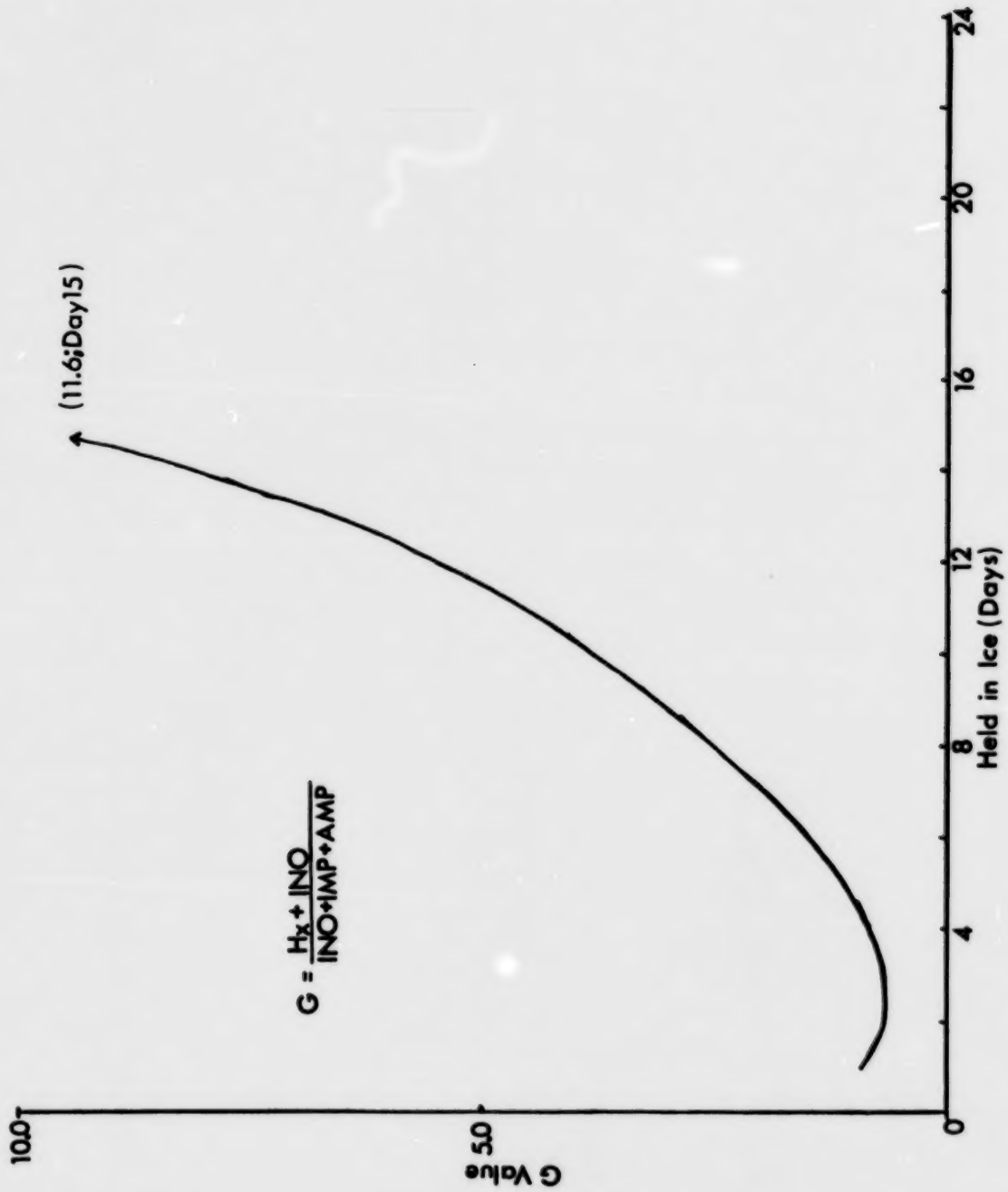


FIGURE 14: G VALUE CHANGES FOR COMMERCIALY CAUGHT COD BLED AND GUTTED AT SEA AND SUBSEQUENTLY HELD ON ICE.

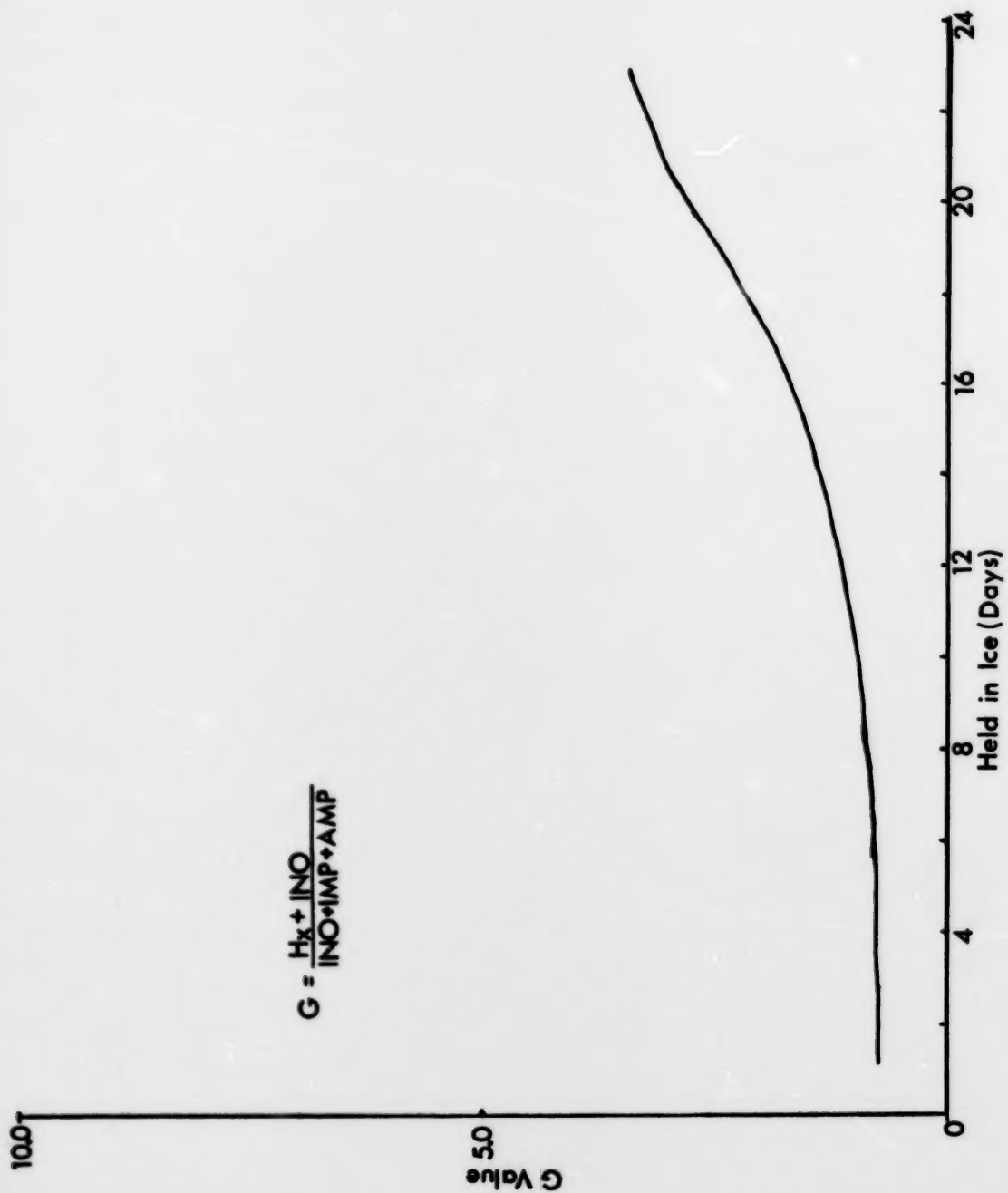


FIGURE 15: G VALUE CHANGES FOR MACKERAL LANDED ROUND THEN GUTTED AND HELD ON ICE

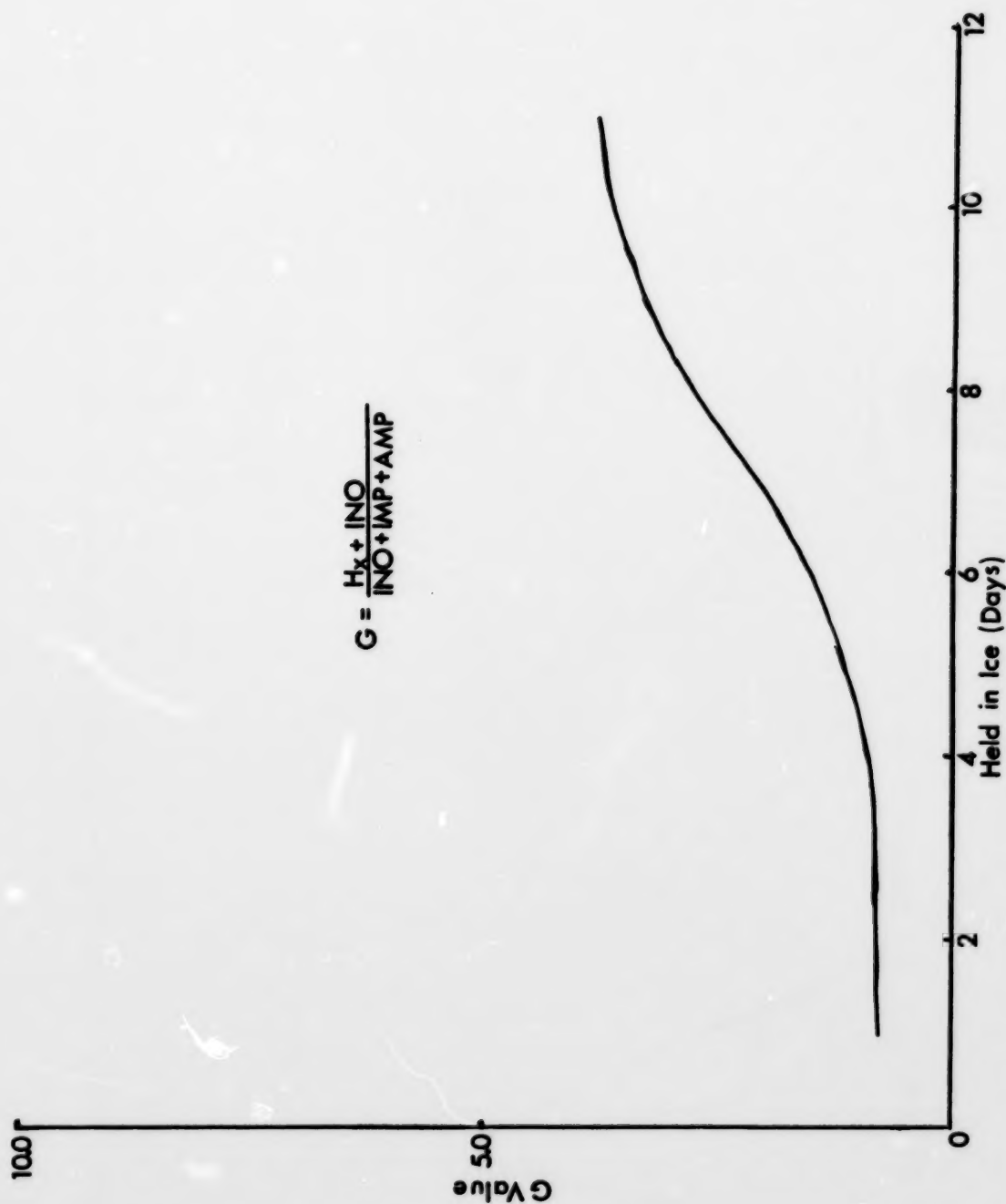


FIGURE 16: G VALUE CHANGES IN SECTIONS FROM LIVE BOTCHED QUEEN CRAB HELD ON ICE.

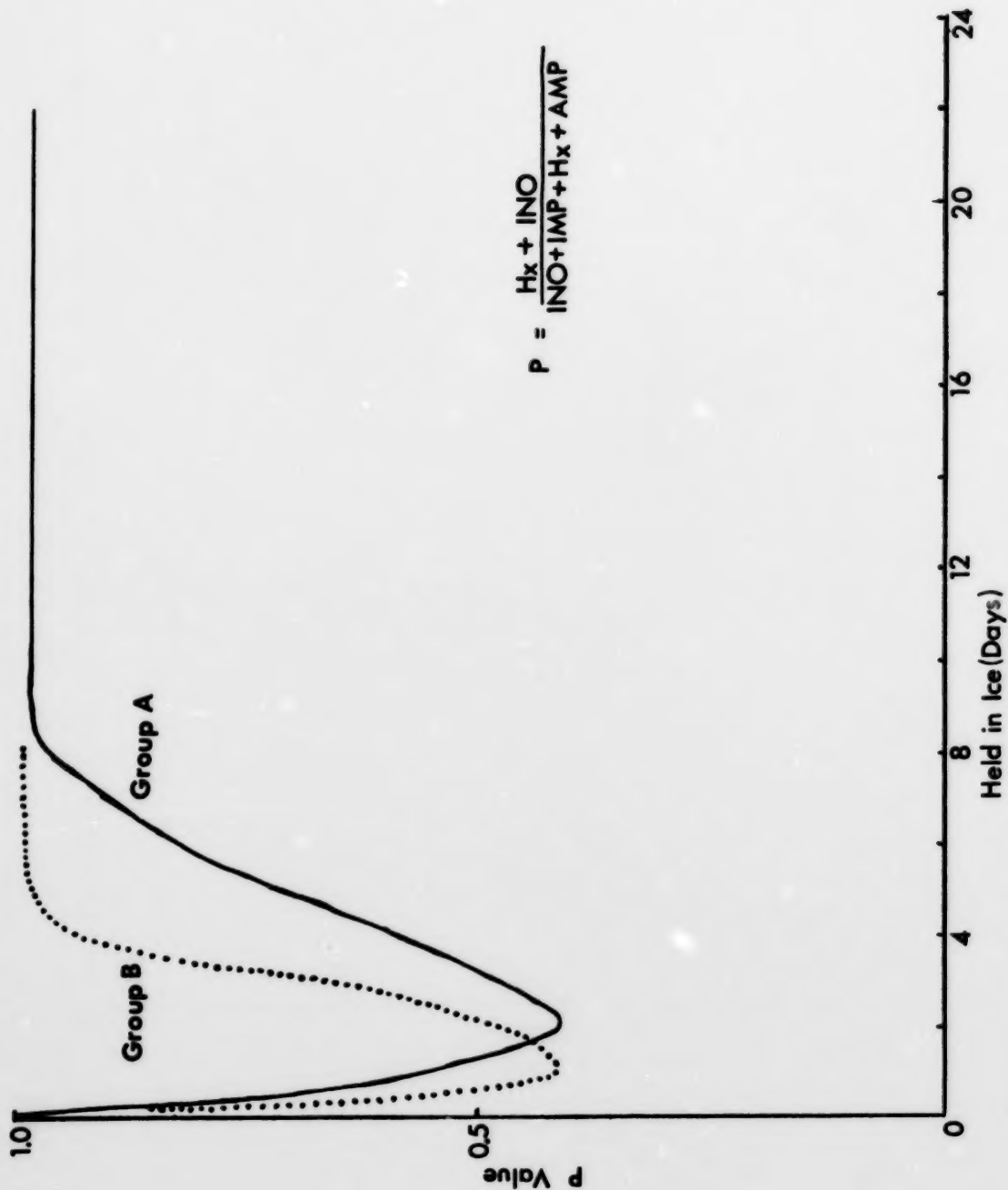


FIGURE 17: P VALUE CHANGES FOR AQUARIUM COD GROUP A; AND GROUP B, BLED, GUTTED AND HELD ON ICE.

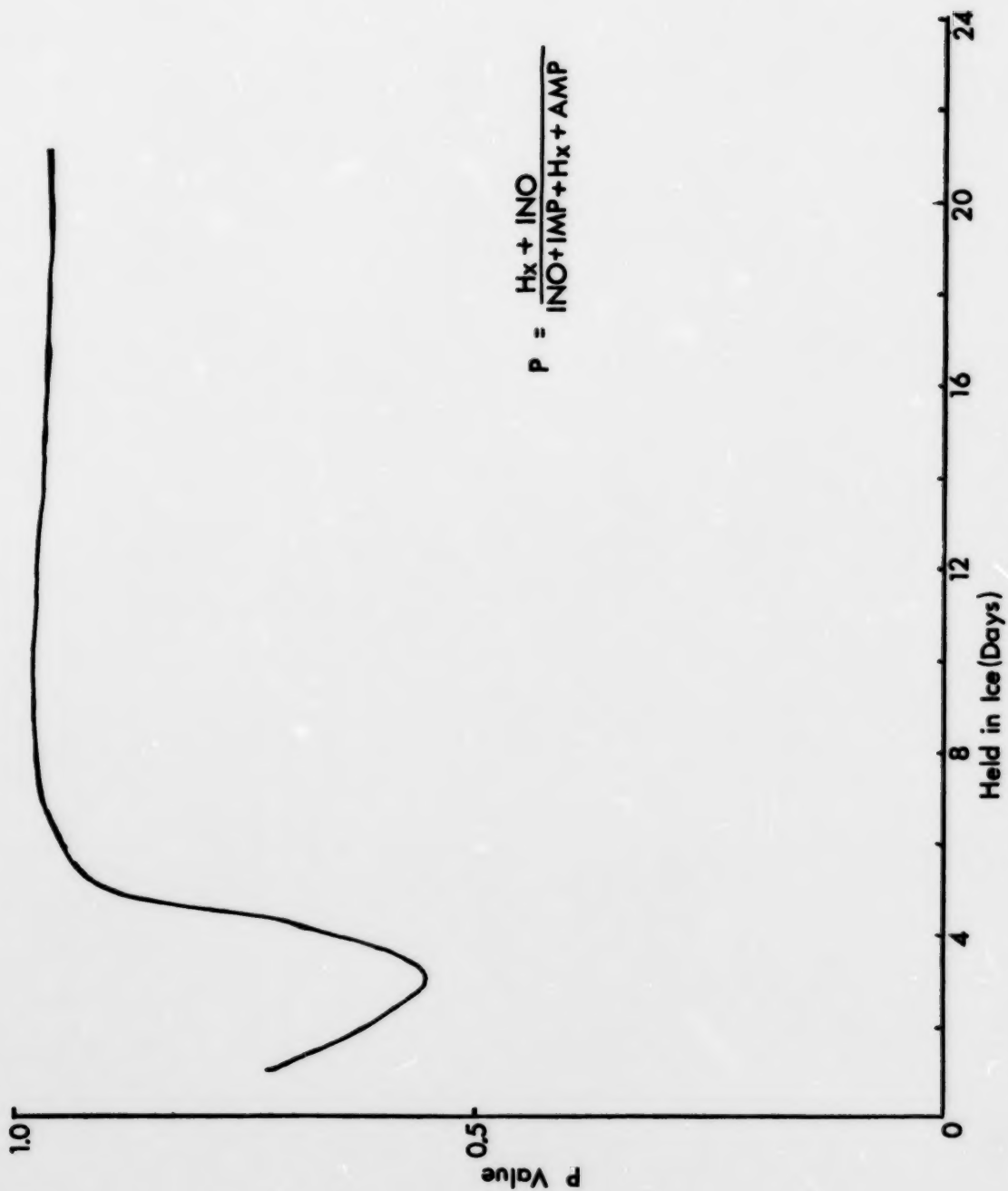


FIGURE 18: P VALUE CHANGES FOR COMMERCIALY CAUGHT COD BLED AND GUTTED AT SEA AND SUBSEQUENTLY HELD ON ICE.

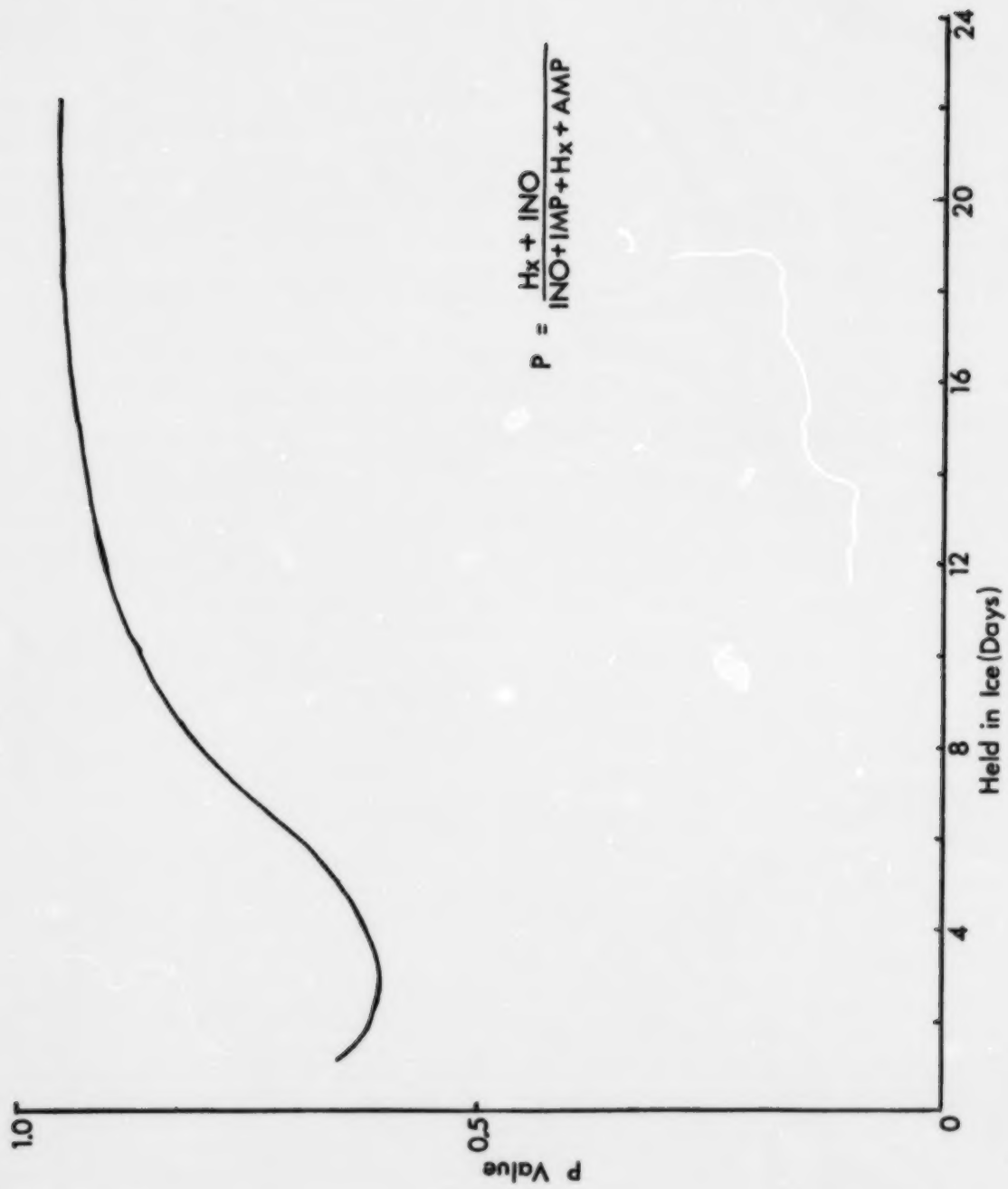


FIGURE 19: P VALUE CHANGES FOR MACKEREL LANDED ROUND THEN GUTTED AND HELD ON ICE.

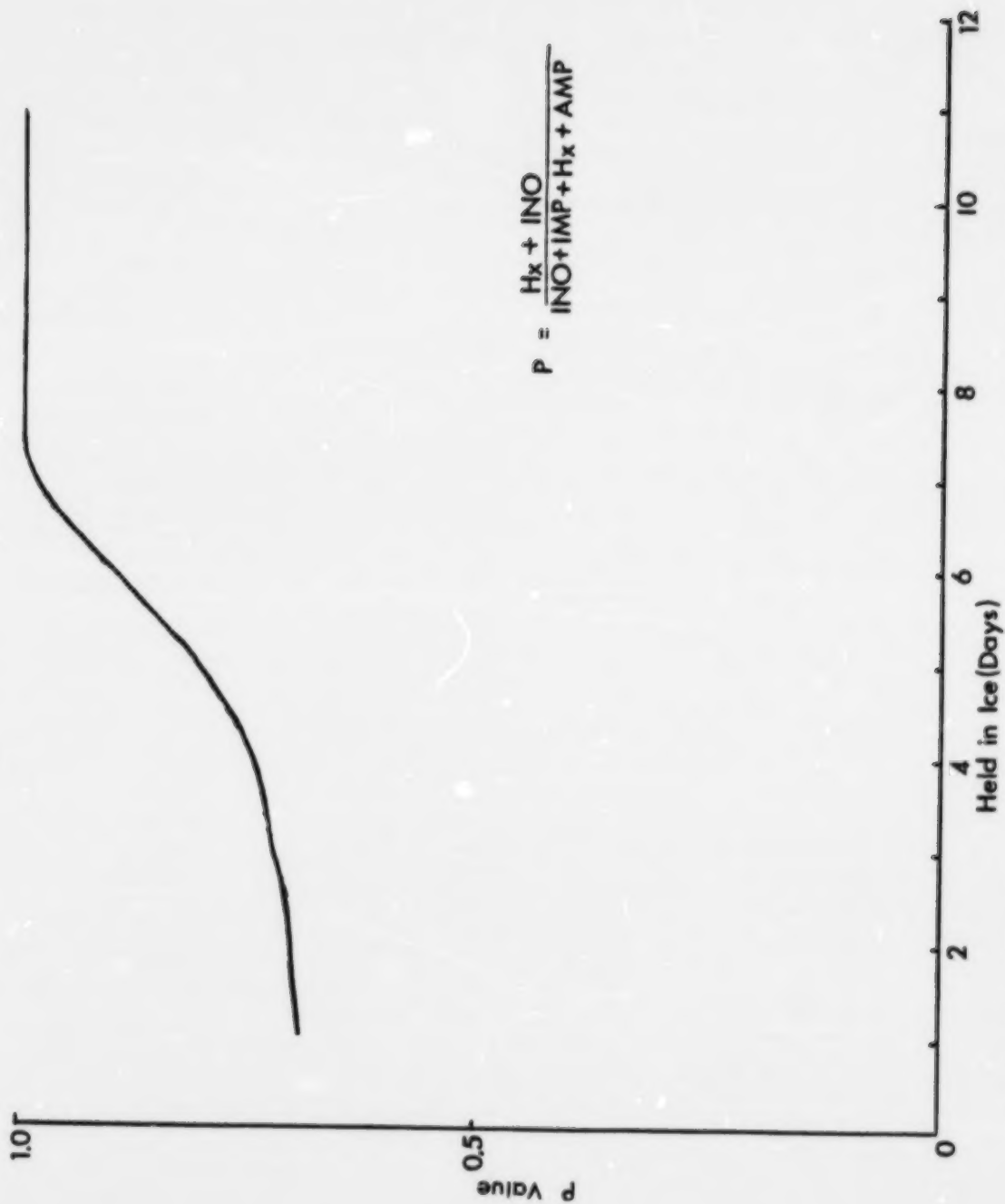


FIGURE 20: P VALUE CHANGES IN SECTIONS FROM LIVE BOTCHED QUEEN CRAB HELD ON ICE.